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MICROCHIP TECHNOLOGY IN MASS SPECTROMETRY-BASED BIOANALYSIS: ADVANCES IN THE ANALYSIS OF PEPTIDES, PROTEINS, AND PHARMACEUTICALS

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ACADEMIC DISSERTATION

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ABSTRACT

The area of microfluidic analysis systems is a rapidly developing field and the hyphenation of chip electrophoresis to mass spectrometry (MS) is a very active research area. Microanalytical techniques are particularly attractive for analysis of biological samples where sample amounts can be extremely low and fast analyses are required. In addition, analytical microsystems provide the prospect of integrating several functional elements on a single platform. The aim of this work was to develop analytical microsystems for fast and efficient analysis of biomolecules and small drug molecules. For this purpose, microchips were fabricated of epoxy photoresist SU-8 using photolithography and adhesive bonding techniques. The free-standing microchips incorporated microchannels for separation and injection, as well as monolithically integrated electrospray ionization (ESI) emitter. The analytes were separated by microchip capillary (zone) electrophoresis (MCE), on-chip capillary isoelectric focusing (cIEF), and cIEF-transient isotachophoresis (tITP) followed by ESI/MS detection.

Peptide mass fingerprinting and protein sequencing were performed for standard proteins. After digestion, the characteristic tryptic peptides were easily separated by MCE and a number of singly or doubly charged peptides were detected by ESI/MS. Sequence coverages between 50% and 70% were routinely obtained for all selected proteins. Additionally, peptide structural characterization and protein identification based on MS/MS fragmentation data of a single tryptic peptide was achieved. Finally, this rapid (total analysis time from sampling to detection less than ten minutes) and reliable protein identification allowed the use of the microchips in the analysis of human muscle cell lysates.

In addition to MCE, cIEF is an attractive technique for separation of zwitterionic molecules (e.g., peptides and proteins) by their isoelectric points (*pI*). In order to facilitate online coupling of cIEF to on-chip ESI/MS a bilateral sheath flow interface or a two-dimensional separation unit was integrated on-chip. Rapid focusing of peptides based on their *pI* values was achieved due to the pH dependent surface charge of the SU-8 polymer. The inherent electroosmotic flow (EOF) taking place in SU-8 microchannels at high pH was exploited to electrokinetic mobilization of the focused pH gradient so that no external pumps were required. In addition, the two-dimensional chip design enables unique separation selectivity for the peptides based on both their *pI* values and intrinsic electrophoretic mobilities by multiplex-cIEF-tITP.

Rapid microchip based metabolic profiling was demonstrated using authentic urine samples from healthy volunteers after intake of tramadol or paracetamol. Six tramadol metabolites and four paracetamol metabolites, including both phase I- and II products, were separated by MCE and

detected by online ESI/MS within 35 s. In addition to metabolic profiling, Michaelis-Menten kinetics was successfully determined for the CYP450-mediated oxidation of bufuralol to 1-hydroxybufuralol.

To better take advantage of the small sample volumes in MCE and to reach better concentration sensitivity sample preconcentration (pretreatment) was integrated on-chip by solid-phase extraction (SPE) and liquid-phase microextraction (LPME). For SPE, a methacrylate monolith zone was firmly anchored at the injection cross of the MCE-ESI/MS microchip by laser induced photopolymerization. The high power laser beam allowed maskless patterning of a precisely defined monolith zone with fast exposure time (here, 7 min). The monolith was selective toward hydrophobic and hydrophilic molecules and enrichment factors as high as 23-fold was obtained with a loading/injection time as short as 25 s. LPME on the other hand, was relatively easy to downscale to the microchip format and to use for low volume applications. In addition, it offered selectivity in the analysis of less polar metabolites so that one tramadol phase I metabolite (M9) was detected only after LPME and not after SPE when conditions were optimized for SPE of very polar phase II metabolites.

In all, these results show that on-chip electrokinetic separations coupled to online ESI/MS are versatile and have great potential of becoming alternatives to the more conventional techniques (i.e., high performance liquid chromatography (HPLC)).

PREFACE

This work was carried out in the Division of Pharmaceutical Chemistry and Technology, Faculty of Pharmacy, University of Helsinki during the years 2008-2014.

First and foremost I would like to thank my supervisors Docent Tiina Sikanen and Professor Risto Kostiainen, for giving me the opportunity to work on this thesis during so many years. I do not think there are many researchers as dedicated and passionate as Tiina! Her endless time, knowledge, and confidence in all results, also the useless ones, are enviable. Without her help and friendship this thesis would never have finished! Likewise, there are not many professors as enthusiastic as Risto. His positive attitude and supportive personality has helped me and many others a lot. In addition, Professor Tapio Kotiaho is irrecoverable in his knowledge in everything that has ever been published in analytical chemistry.

Warm thanks go to my co-authors for their valuable collaboration and contributions: Susanna Laurén and Pia Suvanto at the Department of Materials Science and Engineering, Aalto University who actually went into the clean room and fabricated the chips for me. Thanks also to their Professor Sami Franssila. In addition, big thanks go to Mrs. Maria-Elisa Nordberg for preparing the enzyme kinetics samples and to Dr. Brianda Barrios-Lopez for fabricating the porous polymer monoliths. A special acknowledge goes to Dr. Katariina Vuorensola for introducing me to the academic community in the beginning of my research career and especially for enormous help with teaching duties, I have enjoyed them a lot! Additionally, I want to acknowledge the reviewers, docent Susanne Wiedmer (University of Helsinki) and Professor Pierre Thibault (University of Montreal) for their careful work and insightful comments that led to considerable improvement of this manuscript.

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laughter. I am immensely grateful, too, to my own wonderful family for always supporting me. Overwhelming thanks go to Tommi for his encouragement, love, and amazing understanding during so many years. Finally, Ellen and Agnes, you are the sunshine of my life!

Espoo, February 2015

Nina Nordman

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This doctoral dissertation summarizes the work presented in the following publications:

I Nina Nordman, Tiina Sikanen, Susanna Aura, Santeri Tuomikoski, Katariina Vuorensola, Tapio Kotiaho, Sami Franssila, Risto Kostiainen. Feasibility of SU-8-based capillary electrophoresis–electrospray ionization mass spectrometry microfluidic chips for the analysis of human cell lysates. *Electrophoresis*, **2010**, 31, 3745-3753.

II Nina Nordman, Susanna Laurén, Tapio Kotiaho, Sami Franssila, Risto Kostiainen, Tiina Sikanen. Interfacing microchip isoelectric focusing with on-chip electrospray ionization mass spectrometry. Manuscript **2015**.

III Nina Nordman, Tiina Sikanen, Maria-Elisa Moilanen, Susanna Aura, Tapio Kotiaho, Sami Franssila, Risto Kostiainen. Rapid and sensitive drug metabolism studies by SU-8 microchip capillary electrophoresis–electrospray ionization mass spectrometry. *Journal of Chromatography A*, **2011**, 1218, 739-745.

IV Nina Nordman, Brianda Barrios-Lopez, Susanna Laurén, Pia Suvanto, Tapio Kotiaho, Sami Franssila, Risto Kostiainen, Tiina Sikanen. Shape-Anchored Porous Polymer Monoliths for integrated online solid-phase extraction-microchip electrophoresis-electrospray ionization mass spectrometry. *Electrophoresis,* **2015**, 36, 428-432.

The publications are referred to in the text by their Roman numerals. The original publications are reproduced with the permission of the copyright holders.

Author´s contribution to the publications included in the doctoral thesis:

Publication I

The analytical research plan and the experimental work, excluding the microfabrication, were implemented by the author. The publication was written by the author with contributions from others.

Publication II

The analytical research plan, planning of the microchip designs, and the experimental work, excluding the microfabrication, were implemented by the author. The publication was written by the author with contributions from others.

Publication III

The research plan and the microanalytical experimental work, excluding the microfabrication, were implemented by the author. The enzyme incubations and the enzyme kinetics experiments carried out with the reference method (HPLC) were performed by Mrs. Maria-Elisa Nordberg. The publication was written by the author with contributions from others.

Publication IV

The analytical research plan and the experimental work, excluding the microfabrication, were implemented by the author. The preparation and optimization of the methacrylate monoliths was done by Dr. Brianda Barrios-Lopez. The publication was written by the author with contributions from others.

ABBREVIATIONS AND SYMBOLS

ADME	adsorption, distribution, metabolism, excretion
AN	anolyte
APCI	atmospheric pressure chemical ionization
APPI	atmospheric pressure photoionization
BGE	background electrolyte
BI	buffer inlet
BSA	bovine serum albumin
CA	catolyte
CE	capillary electrophoresis
CGE	capillary gel electrophoresis
CYP	cytochrome P450
cIEF	capillary isoelectric focusing
COC	cycloolefin copolymer
COP	cyclo-olefin copolymer
EC	electrochemical detection
EIE	extracted ion electropherogram
EOF	electroosmotic flow
ESI	electrospray ionization
FAS	field amplified stacking
FL	fluorescence
flt	floating
gnd	grounded
GRAVY	grand average of hydropathicity index
HF	hydrofluoric acid
HLM	human liver microsomes
(HP)LC	high performance liquid chromatography
HV	high voltage
ITP	isotachophoresis
K_m	Michaelis-Menten constant
L	effective channel length
L_t	total channel length
LE	leading electrolyte
LIF	laser-induced fluorescence
LLE	liquid–liquid extraction
LPME	liquid-phase microextraction
LOC	lab-on-a-chip
M1	*O*-desmethyltramadol
M1-Glu	glucuronide of *O*-desmethyltramadol
M5	*N,O*-didesmethyltramadol
M5-Glu	glucuronide of *N,O*-didesmethyltramadol
M7-Glu	glucuronide of 4-hydroxy-ciclohexyl-*N*-desmethyl tramadol

M9	4-oxocyclohexyltramadol.
MALDI	matrix-assisted laser desorption ionization
MCE	microchip capillary (zone) electrophoresis
MEKC	micellar electrokinetic chromatography
MS	mass spectrometry
MS/MS	tandem mass spectrometry
(O)CEC	(open-) channel capillary electrochromatography
Ormocomp®	trademark of a UV-curable hybrid polymer
PC	polycarbonate
PDMS	poly(dimethylsiloxane)
PMMA	poly(methyl methacrylate)
PI	polyimide
pI	isoelectric point
ppm	parts per million
Q-TOF	quadrupole time-of-flight
SDS	sodium dodecyl sulfate
SI	sample inlet
SLI	sheath liquid inlet
SPE	solid-phase extraction
SRM	selected reaction monitoring
SU-8	trademark of an epoxy-based polymer (glycidyl ether of bisphenol A)
SW	sample waste
TE	terminating electrolyte
t_r	migration time
U	voltage
UV	ultraviolet
V_{max}	limiting rate of enzymatic reaction, “maximum velocity”
μ_{app}	apparent mobility
μ_{EOF}	mobility of the electroosmotic flow
μ_{EP}	electrophoretic mobility
µTAS	micro-total analysis systems
2D	two-dimensional
2D-PAGE	two-dimensional polyacrylamide gel electrophoresis

1 INTRODUCTION

All analytical procedures follow specific steps in a well-defined sequence. First, a representative sample is collected from the system or environment to be studied. Usually the sample will need to be processed in some way in order to be compatible with the analytical method. Sample pretreatment may include purification, concentration, and derivatization. The presence of the analyte or analytes of interest is then ascertained, and often the concentration is quantified.

Lab automation has been a primary focus of analytical chemists over the past years to facilitate the integration of experimental steps and increase throughput of sample analysis while simultaneously providing a cost effective platform for sample and data analysis. Traditionally, analyses are performed by mixing relatively large amounts of sample and reagents in test tubes and analyzing the product(s) with an analytical instrument. The time required to analyze a single sample (including sample pretreatment, separation, and detection) is usually relatively long. Additionally, problems arise if the sample is accessible in only very small amount or it is expensive. Here, microchip technology has much to offer. Several functional elements may be integrated onto a single microfluidic platform, enabling the creation of novel devices with unlimited capabilities. The approach is often referred to as a micro-total analysis system (μTAS) or lab-on-a-chip (LOC). The ultimate μTAS or LOC platform is a small, portable device that automatically and rapidly processes very small amounts of samples, following this with relatively fast analysis. Ideally, these compact devices can easily be carried to nearly any site where analysis is needed and can be operated without the need for a trained technician.

Capillary electromigration techniques are a group of analytical methods that have become very popular for microanalytical systems and that are highly suitable for integration in a chip-based format. Capillary electromigration techniques can be divided into capillary electrophoretic techniques, i.e., capillary electrophoresis (CE), capillary gel electrophoresis (CGE), capillary isoelectric focusing (cIEF), and capillary isotachophoresis (ITP) and electrically driven capillary chromatographic techniques, i.e., capillary electrokinetic chromatography (CEC) and micellar electrokinetic chromatography (MEKC). All of these modes can relatively easily be miniaturized and, in miniaturized form, offer promising alternatives for the analysis of a variety of matrices. In principle, a miniaturized on-chip electrophoretic separation can be performed on a microchip with a T-cross layout with electrodes set in buffer reservoirs at the end of the channels. Only a battery is required for generating the required electrical field. Not only are electrophoretic separations easily miniaturized, but unlike chromatographic separation techniques they actually benefit from miniaturization. While the

interactions between the analyte and the stationary phase decrease when miniaturizing chromatography, in microchip capillary electrophoresis (MCE) the advantage lies in the injection scheme. On-chip sample injection is usually performed across the separation channel (rather than directly into the separation channel as in conventional CE), resulting in a very sharp sample zone. The length of the separation channel can then be reduced and less voltage is required to obtain the desired separation performance. Thus, on-chip electrophoretic separations can be realized in seconds with high efficiency and very low sample consumption.

The high selectivity, low detection limits, and good quantitative capability make mass spectrometry (MS) the most important detection method in bio- and pharmaceutical analysis today. Currently, electrospray ionization (ESI)-MS is the ionization method of choice for microfabricated devices, mostly because lower flow rates can be used without loss of sensitivity (i.e., the nanospray regime). Modern microfabrication techniques also enable rapid fabrication of sharp, accurately defined emitters directly integrated with the separation channel in a reproducible manner.

In my work, free-standing, specially constructed SU-8 microchips were tested for high throughput analysis of biomolecules (I, II) and small drug molecules (III, IV). The microchips were equipped for microchip capillary electrophoresis (MCE) (I, III, IV) and cIEF (II) and ESI/MS detection. The microchip methods were shown to be efficient in metabolomics (III), enzyme kinetics (III), and in proteomics research (I, II). In addition, sample pretreatment by liquid-phase microextraction (LPME, III) or solid-phase extraction (SPE, IV) was performed on-chip before separation and detection.

2 REVIEW OF THE LITERATURE

2.1 Toward lab-on-a-chip devices

In response to the need for faster and more cost-effective analytical devices, miniaturization has emerged as a major field of research, extending from basic research to commercial applications. Even though the first example of a miniaturized analytical instrument was a gas chromatograph on a silicon chip demonstrated in the 1970s,[1] it was only in 1990 with the introduction of the μTAS concept that liquid-phase separation techniques[2-6] and coupling to MS[7,8] were introduced. Nowadays, miniaturization and integration of multiple analytical operations on a single microfluidic chip (i.e., LOC technology) is considered to be one of the most promising technologies for the challenging problems of modern bioanalysis.[9-12] Miniaturized analytical devices are desired in highly diverse fields of research, and thus several factors are driving the current interest in miniaturization. In the health care field, analyzers for point-of-care testing need to be small, lightweight, and portable with low power requirements and high batch-to-batch reproducibility. High throughput drug discovery, on the other hand, requires high-density arrays of microvolume reaction vessels for rapid screening of thousands of drug candidates against thousands of biological targets. Likewise, the pressing demands to monitor and detect the release of biological warfare agents on-site drive research in this field. Today, applications of microanalytical systems widely cover clinical diagnostics,[13,14] bioanalytical and pharmaceutical research,[15,16] environmental research,[17,18] cell manipulation and analysis,[19] and proteomics.[20]

A key benefit of miniaturization is the prospect of integrating all steps in an analytical procedure (i.e., sample preparation, concentration, derivatization, injection, separation, and detection) into a single microfluidic device.[21] Many of these standard analytical operations have, in fact, been independently miniaturized and are available as building blocks for fully integrated analyzers.[22] The advantages of such multifunctional devices would include rapid yet sensitive analyses with low consumption of samples and solvents, easy operation, and portability. Microfluidic devices should also provide advantages in terms of analytical performance, i.e., plate numbers, peak capacity, limit of detection, resolution, and so on. For the future, the escalating interest in high-throughput screening for drug discovery, the large-scale analysis required for genetic studies, and the potential of modern microfabrication techniques for mass production of rapid and cost-effective highly integrated devices for parallel analyses will certainly continue to drive and influence further research in miniaturization in general and μTAS in particular.

2.2 Microchip-based electrospray ionization mass spectrometry

The implementation of miniaturized ESI on microchip devices connected online to a mass spectrometer has attracted much interest in recent years. For this, the development of an efficient on-chip interface that transfers dissolved analytes from the microchannel into the gas phase is critical. Although microfluidic devices have been successfully combined with several ionization techniques, including atmospheric pressure chemical ionization (APCI),[23] atmospheric pressure photoionization (APPI),[24] matrix assisted laser desorption ionization (MALDI),[25] and other desorption ionization techniques,[26,27] the predominant approach at present is to exploit the electrospray process. The first step toward miniaturization of ESI was the discovery of the nanospray technique in the mid-90s.[28,29] In nanospray, small droplets are generated from emitters with an inner diameter of just 1–10 μm. This allows reduction in the applicable flow rates (typically down to about 10 nL/min), improved ionization efficiency,[29] and reduced ion suppression.[29,30] The first coupling of electrospray to a microfluidic device was also reported in the 1990s and comprised a glass chip with a channel opening at the edge (Figure 1A).[7,8] Problems were associated with this blunt end approach, however, because of the large dead volumes produced when the droplet spread on the flat surface around the channel exit port. An alternative approach involved attaching external capillaries[31] (Figure 1B) or emitters[32] (Figure 1C) to the microchannel outlet. Despite issues associated with dead volumes in the junction between the (separation) microchannel and the inserted capillary tip and with the poor reproducibility of the fabrication process, combinations including on-chip separations have been successfully achieved with this approach.[33-37] Still, realization of adequate on-chip separations ahead of ESI/MS detection requires fully dead-volume-free interfacing between the separation column or microchannel and the emitter. For this purpose, patterning of the emitter monolithically at the end of the separation microchannel (Figure 1D) is by far the best choice. Current progress in microfabrication techniques has also allowed the development of such sharp-pointed, on-chip emitters for sophisticated microfluidic separation devices in combination with ESI/MS detection.

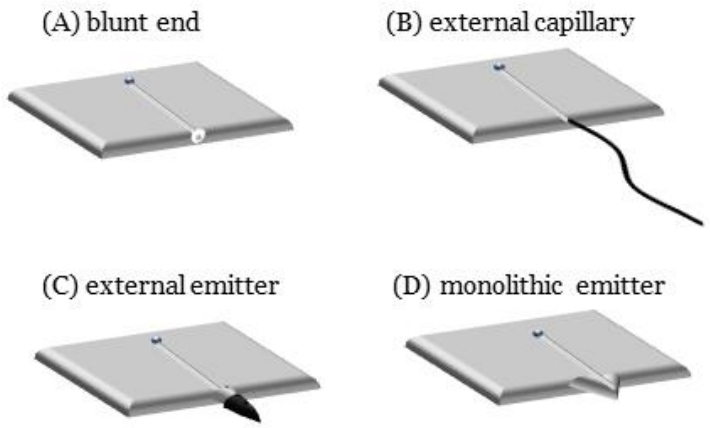


Figure 1 (A-D) Overview of different approaches to combine microfluidic channels with mass spectrometry (MS). Picture adapted from Ref. [38].

Since the microfluidic community has adopted most of their microfabrication processes from the semiconductor industry, silicon was the first material to be used.[3,39] However, the popularity of silicon as the fabrication material rapidly declined, mainly because silicon, as a semiconductor, is not as such compatible with the use of high electric fields for the generation of electroosmotic flow (EOF). The same photolithographic techniques were, however easily adapted to glass and quartz substrates and the interest in these materials grew rapidly.[5,40] Glass is a chemically inert material, has well defined surface characteristics, and can be used to generate EOF. Later on, polymers have gained in popularity as substrate material for microfluidic devices. Especially polymer microfabrication technology offers great opportunities in comparison to silicon and glass.[41] Patterning of polymer substrates is relatively rapid, easy, and inexpensive while accurately defined structures can easily be fabricated as integral parts of these microdevices. Lithographically defined polymer materials, in particular, enable mass production of very complex yet accurately defined microstructures.[42] A wide variety of polymers have thus been used, including poly(methyl methacrylate) (PMMA),[43] polycarbonate (PC),[44] cycloolefin copolymer (COC),[45] polyimide (PI),[46] poly(dimethylsiloxane) (PDMS),[47] SU-8,[48,49] and Ormocer®.[50]

So far, the main effort has been to miniaturize the MS ion source, and microfluidic devices are typically coupled to conventional MS instruments. For truly portable devices, however, focus in the future must also be on the development of miniaturized mass spectrometers. Several research groups are working toward this end and, today, nearly all types of mass analyzers have been miniaturized for the purpose of developing portable MS instruments.[51]

2.3 Microchip capillary electrophoresis and capillary isoelectric focusing

With the electrokinetic liquid handling, relatively easy setup, and no need for pumps or valves, CE has been the separation method of choice for microfluidic separation devices. The chip-based technique is more commonly known as microchip electrophoresis (MCE), though the separation mechanism is basically the same as that in conventional CE. In cathodic mode CE and MCE, positively charged molecules move from the anode to the cathode where they are detected by a suitable detector. The rate of movement of the ionic species is determined by their characteristic electrophoretic mobilities (μ_{EP}), which depend on charge and size of the molecules. The μ_{EP} can be determined experimentally from the migration time and field strengths

$$\mu_{EP} = \frac{L \times L_t}{t_r \times U}$$

where L is the effective capillary/microchannel length (i.e., the distance from the inlet to the detection point), t_r is the migration time of the analyte, U is the applied voltage, and L_t is the total length of the capillary or microchannel. However, because of the EOF caused by the charged substrate surface and the applied electric field, the whole solution (including anions) moves toward the cathode. Hence, the migration of species (apparent mobility, μ_{app}) is determined by the sum of the μ_{EP} and the electroosmotic mobility (μ_{EOF}).

$$\mu_{app} = \mu_{EP} + \mu_{EOF}$$

MCE separation channels are often significantly shorter (usually only a few centimeters) than conventional CE capillaries. In addition, the greater surface/volume ratio and larger thermal mass of the microchip substrate lead to better heat dissipation and the possibility to apply higher separation voltages. This means that improvements in the analytical performance can be obtained not only in faster separations (~10 s to a few minutes) due to shorter channels but also in higher plate numbers (10^5–10^6 m^{-1}) due to higher separation voltages.[52] The setup and functioning principle of a typical LOC system used for MCE is pictured in Figure 2.

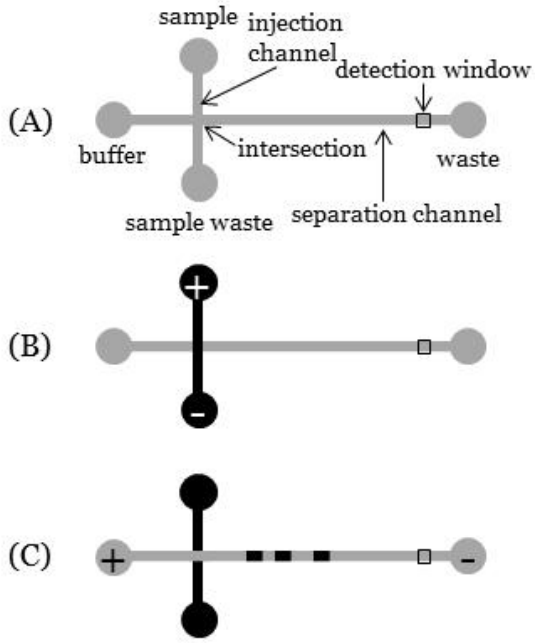


Figure 2 Setup and functioning principle of a typical MCE system. (A) Schematic view of a typical MCE setup with separation and injection channels, liquid reservoirs and detection window, (B) voltage setup during injection, and (C) voltage setup during separation.

Sample injection in microfluidic systems is usually performed electrokinetically through the separation channel (Figure 2B) so that the amount of sample injected (typically in pL range) is determined by the volume of the intersection of the injection and separation channels. Figure 2 shows a microchip with a simple cross-injection structure, but other configurations, e.g., T or double-T injectors, are also common.[53] To avoid broadening of the injected sample plug, "pinched injection" is often implemented. Here, small pushback voltages are applied to the buffer reservoirs so that the sample cannot diffuse into the separation channel during injection. Compared with conventional CE, where injections are directly into the separation capillary (hydrodynamically or electrokinetically), the microchip approach provides very short sample plugs and results in sharp analyte bands and excellent resolution during the following separation. At the same time, the minute sample volumes injected into a microchip electrophoretic system place great demands on the detection sensitivity. Both electrochemical detection (EC)[54] and laser-induced fluorescence (LIF)[55] are widely used with MCE devices. LIF is a powerful technique with sensitivity down to the single-molecule level. A drawback is, however, the need to derivatize most analytes prior to analysis.[55] The ease of miniaturization and integration of EC elements offers a high potential for the development of portable analytical devices. However,

problems of interference of the separation voltage with the detection system, long-term stability of the response, and restrictions regarding the buffer composition still remain a challenge.[54] As MS offers structural information about the analytes and high selectivity, it has become an attractive detection techniques in MCE. In combination with ESI, MS is also a rather universal detection technique allowing label free detection of various ionic and polar compounds. Nevertheless, hyphenation of microfluidics with MS is not an easy task and often means coupling tiny microfluidic chips with huge MS instruments. Figure 1 gives an overview of different approaches to combine microfluidic channels with MS. In terms of minimizing detrimental dead volumes, the preferred approaches (monolithically) integrate the ESI emitter with the separation microchannel (Figure 1D), ideally with the emitter area as small as possible. Table 1 lists MCE separation devices with monolithically integrated ESI emitters developed for the analysis of small molecules and biomolecules.

Table 1. *Selected designs in which ESI-MS tips are monolithically integrated with MCE separation chips.*

Chip material	ESI interface	Analytes	Reference
PDMS	Graphite coated tip	Peptides	[56]
PDMS	Graphite coated tip	Peptides	[57]
SU-8	Sheath flow interface	Drug molecules, peptides, proteins	[48]
SU-8	Sheath flow interface	Peptides, protein digest	I
SU-8	Sheath flow interface	Pharmaceuticals	III
SU-8	Sheath flow interface	Pharmaceuticals	IV
Glass	Sheathless	Pharmaceuticals	[59]
Glass	Sheath flow interface	Peptides, proteins, protein digest	[60]
Glass	Sheath flow interface	Cell lysate	[61]
Glass	Sheath flow interface	Small molecules, proteins	[62]
Cyclo-olefin polymer	Gold coating	Small molecules, amino acids	[58]
Ormocomp polymer	Sheath flow interface	Proteins	[50]

As evident from Table 1, different polymers as substrate material are popular for monolithic integration of MCE separation units with ESI tips.[48,50,56-58] Polymer materials enable rapid and simple chip fabrication at relatively low cost. However, polymers are not always the best substrate material in bioanalysis due to unspecific interactions between the hydrophobic polymer surface and biomolecules (e.g., proteins and peptides). Therefore, alternative ways of fabricating fully microfabricated emitters have been realized also with glass as the substrate material.[59-62] Even though a fully microfabricated glass ESI tip monolithically integrated with a MCE separation unit was presented recently,[62] manual processing steps are often required when fabricating microchips of glass.[59-61] In terms of analytical performance, Thorslund et al.[56] and Dahlin et al.[57] used PDMS chips with a graphite-coated tip for the separation of peptides, accomplishing separations within 2 min on 6-cm[56] and 17-cm-long[57] separation channels, respectively. Plate numbers were of the order of 10^4 m^{-1}. Even higher plate numbers were obtained for the separation of standard peptides by Mellors et al.[60] (10^5–10^6 m^{-1}) using a 4.7-cm-long or 20.5-cm-long serpentine separation channel. Similar high plate numbers and efficient separation were achieved by Sikanen et al.[48] and Sainiemi et al.[62] in their separation of small molecules and peptide standards in 1–2 min on SU-8 and glass MCE-ESI/MS microchips, respectively.

cIEF is an established separation technique for zwitterionic biomolecules (e.g., peptides and proteins), exploiting a unique separation principle based on their isoelectric points (*pI*).[63] In cIEF, carrier ampholytes form a pH gradient under the influence of an electric field, and ampholytic sample ions are separated in sharp bands according to their *pI* values (Figure 3). Like other electrokinetic separation techniques, cIEF is rapid, requires only a small amount of sample, can be automated, and has high separation efficiency. In terms of sensitivity, cIEF is also attractive as the first-dimension separation of a multi-dimensional microchip-based separation device. The focusing nature of cIEF can increase sample concentration up to 100-fold[64] and significantly improve the detection limits for analyses on miniaturized devices.

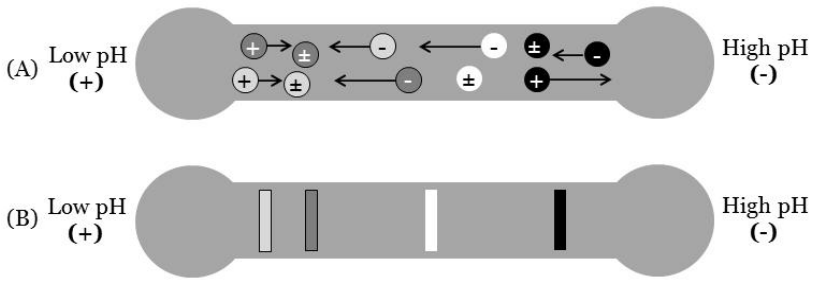


Figure 3 Functioning principle of cIEF. When an electric field is applied between the cathode and the anode (A) ampholytic analytes (e.g., proteins and peptides) move to their isoelectric point where each individual analyte posseses a neutral charge (B).

In cIEF, analyte separation is complete after focusing, but if detection is to be performed with a single-point detector, the focused zones must first be mobilized. The two most common mobilization techniques are hydrodynamic flow and electrophoretic mobilization. Hydrodynamic flow mobilization is usually generated under pressure or gravity, and an electric field is maintained to suppress defocusing of the bands during mobilization.[65] Electrophoretic mobilization can be separated into salt mobilization[66] and EOF-driven mobilization.[67] In salt mobilization, ions are added to one of the electrolytes (cations to the anolyte or anions to the catolyte). The addition of ions induces a pH shift at one end of the cIEF channel, and as this gradually progresses deeper into the separation channel it causes the whole pH gradient/sample solution to migrate toward the anode or cathode. In EOF-driven mobilization, in turn, the whole volume of solution within the channel is driven toward the detector by the EOF inherent in the separation channel.

Microfabricated separation devices for cIEF have primarily relied on ultraviolet (UV) or fluorescence (FL) detection[68-71] in addition to whole column imaging.[72] Although MS detection is often desired, coupling with cIEF is somewhat troublesome because the carrier ampholytes required for successful cIEF tend effectively to suppress the ionization efficiency of the analytes.[73,74] In spite of this, Wen et al.[75] fabricated a polycarbonate microchip for direct coupling of cIEF to ESI/MS and successfully separated and detected standard proteins (Figure 4). Still, the cIEF step (before mobilization) is relatively long (10 min) and a shorter cIEF step would better meet the demands for fast(er) analysis on microfabricated devices.

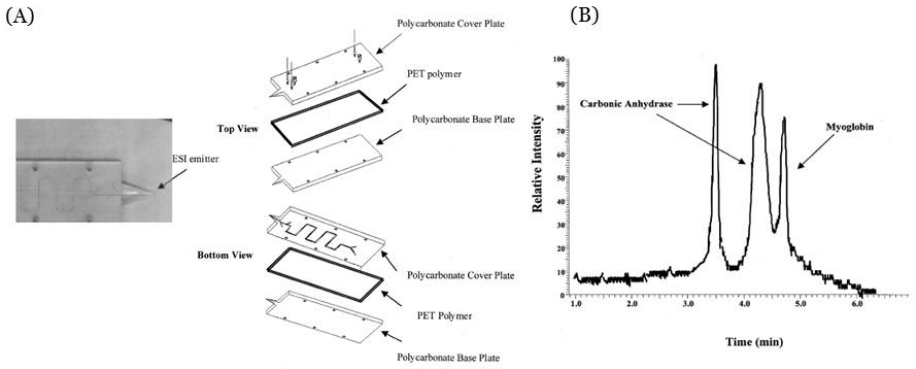


Figure 4 (A) Photograph of the cIEF chip with ESI emitter and expanded view showing the fabrication of a cIEF-ESI/MS microchip. (B) Electropherogram of carbonic anhydrase (*pI* 5.9 and 6.8) and myoglobin (*pI* 7.2) separated by microchip cIEF and detected by ESI/MS.[75] *Reproduced with the permission of John Wiley and Sons.*

2.4 Microchips for multidimensional separations

In most situations, separation is achieved by a single mechanism where the resolution and peak capacity are limited. Multidimensional separation systems are attracting much interest mainly because of the increased peak capacity that they offer over one-dimensional separations.[76,77] Here, microchip technology offers advantages such as high speed of analysis, high throughput, and low sample volume requirements. Sequential coupling of different electrokinetic separations on-chip also does not require carefully designed capillary connectors but is accomplished by proper design of the channel layout. All functional elements are then easily integrated with near-zero dead-volumes on a single monolithically fabricated platform.

The most common multidimensional separation method for protein and peptide analysis is two-dimensional polyacrylamide gel electrophoresis (2D-PAGE).[78] In 2D-PAGE, sample molecules (e.g., proteins) are separated by their *pI* value in the first dimension and molar mass in the second. MS detection (offline) of the spots on the 2D gel enables identification of the separated species. The technique has several limitations, however. It is time-consuming, labor-intensive, and difficult to automate, and throughput is low.[79] 2D-PAGE has also been successfully miniaturized.[80-82] On the other hand, liquid-phase separations are more convenient for online MS coupling. Table 2 lists microchip-based 2D separation devices with in-solution separations, including both chromatographic and electrophoretic approaches.

One of the first in-solution 2D separation microchip was introduced by the Ramsey group in 2000 (Table 2).[83] They separated peptides by first-dimensional MEKC, using sodium dodecyl sulfate (SDS) as the micellar dispersed phase, and second-dimensional MCE in less than 10 min, following this with FL detection. Both the resolving power and peak capacity were greatly increased over values obtained by either dimension alone. In terms of sample throughput, analysis with the MEKC-MCE microchip is the fastest thus far reported for 2D microchip separation systems (Table 2).

Table 2. *Summary of selected in-solution-based 2-dimensional microfluidic separation devices.*

Separation technique	Analysis time	Detection method	Peak capacity	Reference
MEKC × MCE				
Peptides	8-10 min	FL	500-1000	[83]
Tryptic peptides	10-15 min	FL	4200	[84]
Tryptic peptides	10-35 min	FL	>4000	[85]
OCEC × MCE				
Tryptic peptides	10-15 min	FL	150	[86]
cIEF × MCE				
Proteins	*n* × 45 s	FL	1300	[87]
Proteins	20 min	FL	not reported	[88]
Tryptic peptides	50 min	FL	540	[89]
LC × MCE				
Tryptic peptides	>30 min	ESI/MS	30-fold compared to 1D	[90]
cIEF × LC				
Tryptic peptides	60 min	FL MALDI/MS	215	[91]
cIEF × tITP				
Peptides	<10 min	ESI/MS	not reported	II

2.5 On-chip sample pretreatment

A well-known problem today, particularly in on-chip electrophoretic separations, is the relatively poor concentration sensitivity, due to the short optical path length in UV and FL detection and the extremely small amount of sample injected. For ESI/MS, detection analysis from subattomole amounts of sample has been demonstrated,[31,92] but the very small injection volumes (typically in pL range) often force the use of micromolar concentrations if there is no preconcentration step. Additionally, sample pretreatment (and simultaneous preconcentration) is usually considered to be the most time-consuming step in bioanalyses. Thus, extensive work has been carried out with a view to integrating this step with microchip electrophoresis systems.[93]

In general, chip-based sample pretreatment methods are analogues of their conventional counterparts and fall into one of four major categories, as indicated in Figure 5. In this overview, I consider only on-chip sample preconcentration, and thus, only the electrokinetic techniques (field amplified stacking and isotachophoresis) and extraction-based techniques (SPE and liquid–liquid extraction (LLE)). Additionally, concentration enhancement factors up to 30 and 100 have been reported for on-chip

sample preconcentration by surface affinity reactions[94] and various membrane-based techniques,[95] respectively.

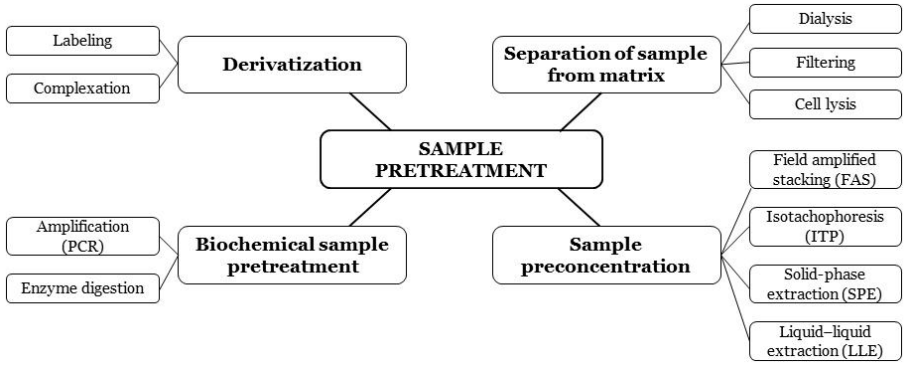


Figure 5 Classification of chip-based sample pretreatment techniques. Picture adapted from Ref. [96].

2.5.1 Electrokinetic techniques

One of the most common techniques for increasing the concentration of desired compounds is field amplified stacking (FAS). FAS was first used on a microfluidic device in 1995,[97] when amino acids were separated by electrophoresis. In a typical sample stacking approach, the analyte in a low conductivity buffer is injected into the separation channel (capillary or microchannel) between higher ionic strength running buffers. After applying voltage, analytes accelerate toward the cathode/anode due to the faster migration in the lower ionic strength sample solution (high field strength). When they reach the sample zone/BGE boundary they are slowed down (low field strength) and become stacked into narrow bands. The sample ions become stacked at the interface between the low and high conductivity buffers owing to the higher electric field strength in the low conductivity buffer. FAS has also been applied for the concentration and MCE separation of fluorescently labeled amino acids[98] and for MCE and MS detection of trace level digests of gel-isolated proteins.[99] Here, the concentration enhancements were 95-[98] and 50-fold,[99] respectively. The simplicity, easy applicability, and compatibility with different buffers make FAS widely applicable to many different detection systems, including MS.[99]

ITP is another well-established technique that can be used both for sample preconcentration and as an individual separation technique. In ITP the sample is injected between a leading electrolyte (LE) and a terminating electrolyte (TE) so that the electrophoretic mobility of the LE ($\mu_{EP}(LE)$) is greater than the maximum μ_{EP} in the sample and, reversely, the $\mu_{EP}(TE)$ is lower than the minimum μ_{EP} of the sample. When an electric field is applied, the sample ions are separated in very sharp bands between the LE and the TE

in the order of their μ_{EP} values, and the zones migrate with uniform velocity toward the detection point (Figure 6).

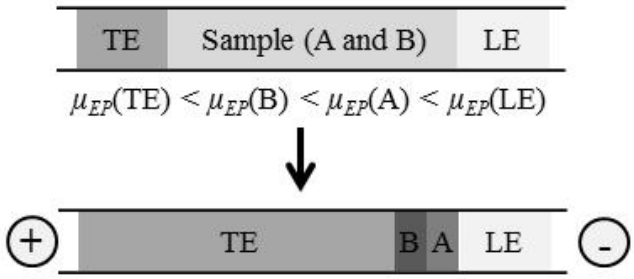


Figure 6 Functioning principle of ITP. The sample solution (A and B) is introduced between a leading electrolyte (LE) possessing higher electrophoretic mobility (μ_{EP}) than A and B and a terminating electrolyte (TE) possessing slower μ_{EP} than A and B. Upon application of an electric field between the anode and the cathode A and B separate in sharp bands between the LE and the TE.

ITP provides a simple and powerful method of concentration that can easily be integrated to the microchip format ahead of other on-chip operations. It can provide an increase in sensitivity of several orders of magnitude for dilute samples, while also being suitable for highly saline samples. ITP has already been extensively used as a preconcentration method in MCE,[100] and sample enhancement factors as high as 800 have been achieved.[101] One drawback of ITP is that it is usually necessary to determine the μ_{EP} of sample ions before the analysis. This may be time-consuming and troublesome especially for complex samples with many analytes of interest.

2.5.2 Extraction techniques

Common to all electrokinetic techniques is that one must fill the microchannel with two or more different buffer solutions to create appropriate electric field gradients for sample stacking and concentration. Furthermore, electrokinetic stacking methods are not very suitable for sample clean up (e.g., removing salts from the matrix) and additional off-line sample pretreatment is often required for very complex samples. Moreover, electrokinetic approaches typically deliver enrichment factors an order of magnitude lower than those of the more effective extraction-based techniques.[96]

Chip-based extractions have been performed by both SPE and LLE.[96] SPE relies on interactions between analytes (in mobile phase) and a solid-phase packing (stationary phase), which under the appropriate conditions, result in retention of analytes onto the stationary phase. The choice of stationary phase material allows the SPE device to be tailored between the

great selectivity achievable with an immunoaffinity ligand and the completely generic result achieved with the octadecyl carbon chain (C18)-bonded silica. SPE is not only a preconcentration technique, but can at the same time be used as a precleaning process for the separation of hydrophobic analytes from hydrophilic impurities, or *vice versa*.

Accomplishing SPE on a microchip requires that, solid support structures are incorporated into the microchannel.[102,103] Beads have been used in analytical devices (e.g., chromatography columns) for several decades and the use of beads in microchannels is also widespread.[104] Trapping beads inside a microchannel for SPE purposes, however, requires some form of immobilization within the device. A number of immobilization methods have been reported, some relying on mechanical barriers, such as microfabricated frits,[47,105] to retain the beads, others on surface immobilization[106] or magnetic fields to manipulate and trap the (magnetic) beads.[107] Precisely ordered microfabricated structures (micropillars), mimicking a perfectly ordered packed bed, have been investigated as alternative stationary phases.[108-110] Ordered pillar arrays are only support structures, however, and surface chemical reactions often must be employed to put the stationary phase material onto the pillars. As an alternative to packed beads, porous polymer monoliths offer attractive features for on-chip SPE. Monoliths are easily formed by *in situ* polymerization of monomers, in the presence of a porogen, by heat or UV. The porosity and surface chemistry of the monolith can be tuned simply by changing the polymerization time and/or monomer composition. Additionally, UV-initiated polymerization methods allow monoliths to be fabricated in specific areas of a microchannel without the need for frits and with no risk of beads leaking out of the microchannel and into the MS instrument. Monoliths are typically silica- or polymer-based and have higher permeability and better mass transfer than comparable particulate materials. This means that they can be run under lower pressure and operated fully electrokinetically. Even if polymerization conditions might be difficult to keep constant during fabrication of monolithic stationary phases, studies have demonstrated that column-to-column reproducibility is not poorer for monolithic stationary phases than for their packed counterparts.[111] Table 3 lists selected microchips with integrated SPE phases for sample pretreatment and/or preconcentration prior to analysis. Table 3 is not comprehensive and does not list packed chips used for e.g., enzyme immobilization.

Table 3. *Selected microchips with integrated on-chip SPE.*

SPE material	Actuation mode	Loading time	Enrichment factor	Separation mode	Detection mode	Reference
Methacrylate monolith	Pumping	>1 h*	10^3	No separation	FL	[112]
Methacrylate monolith	Pumping	0.5 min*	No information	No separation	MS	[113]
Methacrylate monolith	Pumping	>100 min*	267	No separation	MS	[114]
Methacrylate monolith	Electrokinetic	1.7 min	200	No separation	FL	[115]
Methacrylate monolithic	Electrokinetic	5 min	10^5	MCE	FL	[116]
Methacrylate monolith	Pumping	1.5 min	150	On-chip LC	FL	[117]
Methacrylate monolithic	Electrokinetic	20 min	80	MCE	Amperometric	[118]
Methacrylate monolithic	Electrokinetic	30 min	105	MCE	MS	[119]
Methacrylate monolith	Electrokinetic	15 min	>100	No separation	FL	[120][121]
Acrylate monolith	Electrokinetic	2 min	270	CEC	FL	[122]
Methacrylate monolith	Electrokinetic	1-5 min	6-11	MCE	FL	[123]
Methacrylate monolithic	Electrokinetic	0.4 min	23	MCE	MS	IV
ODS-coated silica beads	Electrokinetic	1-3 min	900	MCE	FL	[124]
ODS –or IMAC^ beads. Immobilized trypsin.	Pumping	6 min*	10-20	MCE	MS	[125] [126]
ODS-coated silica beads	Electrokinetic	2 min	500	No separation	FL	[127]
Copolymer† beads	Pumping	No information	80	Off-line CE	UV	[128]
Polystyrene beads	Pumping	2–5 min	No information	MCE	MS	[129]
Octadecylsilane coating	Electrokinetic	1-5 min	80	No separation	FL	[130]
ODS-coated magnetic particles	Electrokinetic	No information	No information	MCE	FL	[107]

*A specific sample volume was loaded. ^immobilized metal affinity chromatography. †Commercial Oasis HLB.

Another extraction technique that has been used extensively for preconcentration in analytical processes is LLE. In microfluidic systems the laminar flow regime can be exploited in order to incorporate LLE on-chip. The possibility of creating large interfacial contact regions relative to the small volumes being manipulated and, short diffusion distances is advantageous in LLE. This straightforward approach of forming a multi-phase parallel laminar flow has been exploited by many groups.[131-133] In these continuous systems, however, enrichment factors higher than 10 are difficult to achieve because of limitations in applicable organic/aqueous phase ratios.

In droplet-based extraction, on the other hand, the volume of the acceptor phase (droplet) can be minimized so that the enrichment from a continuous phase can effectively be achieved.[134,135] At best, enrichment factors higher than 1000 have been achieved with droplet-based microextraction.[134,136]

Although the above-mentioned two-phase techniques are relatively easy to implement even on microchip devices, LLE as such is not easily coupled to MCE separations, primarily due to the often incompatible nature of solvents when extraction is from a water-based solution into an organic phase. This difficulty can be overcome by performing a three-phase extraction in which analytes pass from the sample (donor solution) through an organic phase into an acceptor solution. The organic phase can be held in a membrane (LPME), as has been shown by Pedersen-Bjergaard and co-workers[137-145] for off-line cleanup before conventional CE separations. The setup was further introduced to an MCE glass microchip for LPME before separation and FL detection of pharmaceuticals.[146] LPME is an equilibrium extraction technique where recovery is determined by the partition coefficients of the analytes, the sample volume, the volume of the supported liquid membrane, and the volume of the acceptor phase. At a certain point, the system enters equilibrium and the analyte concentration in the acceptor solution remains constant. Typically, LPME recoveries range between 10 and 90%.[142,146] In another three-phase approach, microdroplets with designer geometries were used in an LLE system.[147] Here, a multiphase droplet with an organic phase sandwiched between two aqueous phases was applied. Fluorescein was then extracted from the aqueous donor droplet solution through the organic phase (octanol) into the aqueous acceptor solution.

3 AIMS OF THE STUDY

The primary aim of the study was to develop fast and efficient analytical microsystems for the analysis of biomolecules and small drug molecules. The various analytes were to be separated on-chip by different electrokinetic separation methods, ionized by on-chip ESI and detected by MS. For this purpose, two microchip designs were reproduced from SU-8 polymer, with all structures simultaneously patterned by photolithography. A further aim was to incorporate sample pretreatment (preconcentration) as an integral part of the analytical microsystem. This was pursued by on-chip SPE and LPME.

The more detailed aims of the research were

- To confirm the applicability of SU-8-based MCE/cIEF-ESI/MS microchips to the very fast characterization of standard proteins and protein mixtures (I, II)
- To solve the challenges related to online coupling of cIEF to ESI/MS by exploiting SU-8 photolithography for chip fabrication (II)
- To take advantage of the pH dependent surface charge of SU-8 polymer in order to perform on-chip cIEF without EOF reductive coating (II)
- To address the need of increased analysis speed in drug development by developing a fast microchip based separation method for analyzing metabolites in human urine samples and determining kinetic parameters (III)
- To couple LPME online to a separation microchip for the precleaning and preconcentration of drug metabolites from human urine (III)
- To perform fast on-chip SPE before MCE-ESI/MS without substantially increasing the total analysis time (IV)

4 EXPERIMENTAL

This section briefly describes the chemicals, materials, samples, instrumentation, and experimental setups used in the work. Details of the experimental conditions and chemicals can be found in the original publications I-IV.

4.1 Chemicals, materials, and samples

Chemicals used in the work are listed in Table 4. Notes indicate their use. All chemicals and solvents were of analytical grade or higher.

Table 4. *Chemicals used in the work*

Reagent/Solvent/Standard	Manufacturer/Supplier	Note	Publication
Acetic acid	Mallinckrodt Baker, Deventer, The Netherlands	Reagent	I-IV
Acetonitrile (ACN)	Rathburn Chemicals, Walkerburn, Scotland	Solvent	I, III
Ammonium acetate	Sigma-Aldrich, Steinheim, Germany	Reagent	I-IV
Ammonium formate	Sigma-Aldrich, Steinheim, Germany	Reagent	I, III
Ampholyte pH 3-10	Sigma-Aldrich, Steinheim, Germany	Reagent	II
Angiotensin I	Sigma-Aldrich, Steinheim, Germany	Standard	I, II
Angiotensin II	Sigma-Aldrich, Steinheim, Germany	Standard	I, II
Angiotensin III	Sigma-Aldrich, Steinheim, Germany	Standard	II
Benzoin methyl ether (BME)	Sigma-Aldrich, Steinheim, Germany	Photoinitiator	IV
Bovine serum albumin (BSA)	Sigma-Aldrich, Steinheim, Germany	Standard	I
Bradykinin (fragment 1-5)	Sigma-Aldrich, Steinheim, Germany	Standard	I, II
Bufuralol	Roche, Basel, Switzerland	Standard	III
Cotinine	Sigma-Aldrich, Steinheim, Germany	Standard	IV
Cytochrome *c*	Sigma-Aldrich, Steinheim, Germany	Standard	I, II
2,3-Epoxypropyl methacrylate (GMA)	Sigma-Aldrich, Steinheim, Germany	Monomer	IV
Fluorescein-polyethyleneglycol (PEG-NHS- 5k)	Creative PEGWorks, Winston Salem, NC, USA	Porogen	IV
Formic acid	Sigma-Aldrich, Steinheim, Germany	Reagent	I, III
Histidine	Sigma-Aldrich, Steinheim, Germany	Standard	II
Human liver microsomes (HLM)	BD Gentest, Erembodegem, Belgium	Reagent	III
Human muscle cell lysate	Protein Chemistry Core Facility, University of Helsinki, Finland	Biological sample	I
Hydrochloric acid	Riedel-de Haën, Seelze, Germany	Solvent	III

Reagent/Solvent/Standard	Manufacturer/Supplier	Note	Publication
Hydrogen fluoride (HF)	Merck, Darmstadt, Germany	Solvent	I-IV
1-Hydroxy bufuralol	Ultrafine Chemicals, Manchester, England	Standard	III
ß-Lactoglobulin	Sigma-Aldrich, Steinheim, Germany	Standard	I
Methanol	J.T. Baker, Deventer, Holland	Solvent	I-IV
1-Methoxy-2-propyl acetate (PMA)	Sigma-Aldrich, Steinheim, Germany	Solvent	III
2-Methoxyethanol (2ME)	Sigma-Aldrich, Steinheim, Germany	Solvent	IV
1-Methyl-2-pyrrolidone (NMP)	Sigma-Aldrich, Steinheim, Germany	Co-porogen	IV
Milli-Q water, deionized	Milli-Q water purification system	Solvent	I-IV
Nicotinamide adenine dinucleotide phosphate oxidase (NADPH)	Sigma-Aldrich, Steinheim, Germany	Cosubstrate	III
N,N,N,N´,N´-Tetramethylethylenediamine (TEMED)	Sigma-Aldrich, Steinheim, Germany	Solvent	II
N,O-Didesmethyltramadol	Department of Forensic Medicine, University of Helsinki, Finland	Standard	III
1-Octanol	Fluka, Buchs, Switzerland	Solvent	III
Ovalbumin	Sigma-Aldrich, Steinheim, Germany	Standard	I
O-Desmethyltramadol	Department of Forensic Medicine, University of Helsinki, Finland	Standard	III
Paracetamol	Orion Pharma, Espoo, Finland	Standard	III
Polyethyleneglycol 6000 (PEG (6k))	Applichem, Darmstadt, Germany	Porogen	IV
Paracetamol glucuronide	Sigma-Aldrich, Steinheim, Germany	Standard	III
Propranolol	Sigma-Aldrich, Steinheim, Germany	Standard	IV
Sodium hydroxide	J.T. Baker, Deventer, Holland	Reagent	III
Sodium phosphate	Riedel-de Haën, Seelze, Germany	Reagent	III
Substance P (fragments 6-11)	Sigma-Aldrich, Steinheim, Germany	Standard	II
Tramadol	Department of Forensic Medicine, University of Helsinki, Finland	Standard	III
Triethylene glycol dimethacrylate (TEGDMA)	Sigma-Aldrich, Steinheim, Germany	Crosslinker	IV
Trimethylolpropane trimethacrylate (TRIM)	Sigma-Aldrich, Steinheim, Germany	Crosslinker	IV
Trizma base	Sigma-Aldrich, Steinheim, Germany	Reagent	I, II
Trypsin (porcine)	Promega, Madison, WI, USA	Enzyme	I, II
Verapamil hydrochloride	Sigma-Aldrich, Steinheim, Germany	Standard	III, IV

Table 5 lists the commercially available materials and products that were used. Notes indicate their use.

Table 5. *Commercially available materials and products used in the study.*

Materials/Products	Manufacturer/Supplier	Note	Publication
Celgard 2500 microporous polypropylene membrane	Celgard, Charlotte, NC, USA	For LPME	III
Isolute MF C18, 100 mg reversed-phase cartridge	Int. Sorbent Technology, Mid Glamorgan, U.K.	For SPE	III
Oasis HBL, 30 mg cartridge	Waters, Milford, MA, ÙSA	For SPE	III
Platinum (999, 0.5 mm)	Kultakeskus, Hämeenlinna, Finland	Electrode material	I-IV
Poly(dimethylsiloxane), Sylgard 184 PDMS	Dow Corning, Midland, MI, USA	Supporting structures for microchips	I-IV
SU-8 photoresist	Microchem, Newton, MA, USA	Fabrication of microchips	I-IV

The drug molecules, proteins, and peptides were dissolved in methanol, water, or water/methanol and further diluted as stated in the original publications (I-IV). Protein digests and human muscle cell lysates (I, II) were prepared according to standard protocols and analyzed without further pretreatment.

The urine samples (III) were pretreated by SPE using ODS reversed phase cartridges (Isolute MF C18, urine containing paracetamol metabolites) or polymeric reversed phase cartridges (Oasis HBL, urine containing tramadol metabolites). In addition to SPE, the urine samples were pretreated by LPME, as described in publication III and in Ref. [146].

4.2 Instrumentation

Table 6 lists the commercial instruments used in the work. Notes indicate their use. In addition to the commercial instruments, some in-house built and standard laboratory equipment was employed (i.e., chip holders, electric resistors, vacuum pumps, microscopes, and multimeters).

Table 6. *Commercial instruments used in the study*

Instrumentation	Manufacturer/Supplier	Note	Publication
For microchip electrophoresis			
Microfluidic ToolKit, computer-controlled (Labview)	Micralyne, Edmonton, Canada	High voltage power supply	I-IV
For monolith preparation			
Zeiss Axioscope	Carl Zeiss, Espoo, Finland	UV laser	IV
For mass spectrometry			
Agilent 6330 MS ion trap	Agilent Technologies, Santa Clara, CA, USA	Mass spectrometer	I, II
Compressed air	Atlas Copco air dryer, Wilrijk, Belgium	Nebulizer gas	I, II
Nitrogen	Nitrogen generator system, Parker, Cleveland, OH, USA	Nitrogen generator	I, II
Nitrogen	Whatman 75-72 nitrogen generator, Haverhill, MA, USA	Nitrogen generator	I, II
Nitrogen	Peak Scientific, Renfrewshire, Scotland	Nitrogen generator	I, II
PE Sciex API 365	Perkin Elmer Sciex, Concord, Canada	Mass spectrometer	II
PE Sciex API 3000	Perkin Elmer Sciex, Concord, Canada	Mass spectrometer	I, III, IV
Q-TOF Micro MS	Waters/Micromass, Manchester, U.K.	Mass spectrometer	I
For reference methods			
Agilent HP 1100 HPLC system	Agilent Technologies, Waldbronn, Germany	Liquid chromatograph	III
AtlantisTM dC18 column	Waters Corporation, Milford, MA, USA	Reversed-phase column	III
Fused silica capillary	Composite Metal Services, Worchester, UK	For capillary electrophoresis	II
P/ACE MDQ CE equipment	Beckman Instruments, CA, USA	Capillary electrophores	II
Zorbax Eclipse Plus C18 column	Agilent Technologies, Palo Alto, CA, USA	Reversed-phase column	III
Software etc.			
Analyst 1.4		Software for Sciex QqQ	I-IV
Data Analysis		Software for ion trap	I, II
Mass Lynx 4.0		Software for Q-TOF	I
Swiss Prot database	MASCOT® online search engine (www.matrixscience.com)	Online protein search engine	I, II

4.3 Microchip design and fabrication

The microchip designs are schematically presented in Figure 7. The dimensions of the microchannels varied, as reported in the original publications (I-IV), but the fabrication process was similar for all designs, comprising standard photolithographic and adhesive bonding techniques.[48,148]

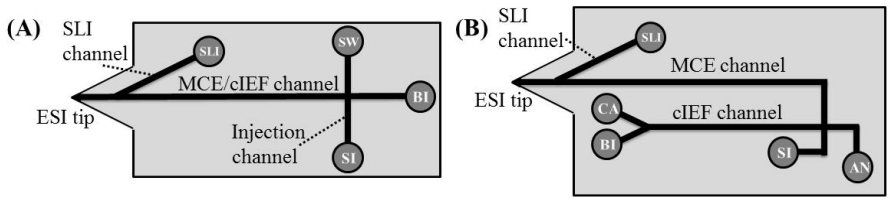


Figure 7 Schematic pictures of the microchips used for (A) MCE and cIEF (for cIEF the microchip had two sheath flow channels) and for (B) cIEF-transient ITP-ESI/MS. AN anolyte, BI buffer inlet, CA catolyte, SI sample inlet, SLI sheath liquid inlet, and SW sample. Dimensions not to scale.

Figure 8 shows the three structural SU-8 layers used in fabrication of the MCE-ESI/MS microchip (Figure 7A). The first SU-8 layer (70-µm-thick) was spin coated on a silicon wafer, and sample inlets were patterned on it. A second (50-µm-thick) SU-8 layer was then spin coated on top of the first and patterned with the microchannels. After post-exposure bake the first two layers were simultaneously developed. The third SU-8 layer (70-µm-thick) was individually spin coated on a polymer substrate, post-baked, and then bonded with the previously patterned SU-8 layers. Exposure of the third SU-8 layer was done through the transparent substrate, and a post-exposure bake followed. Finally, the three-layered microchips were released from the silicon substrate by sacrificial wet etching in 50% HF in water. Before use, poly(dimethylsiloxane) (PDMS) sheets with 2 mm inlet holes were attached on top of the SU-8 chips to increase the inlet volumes.

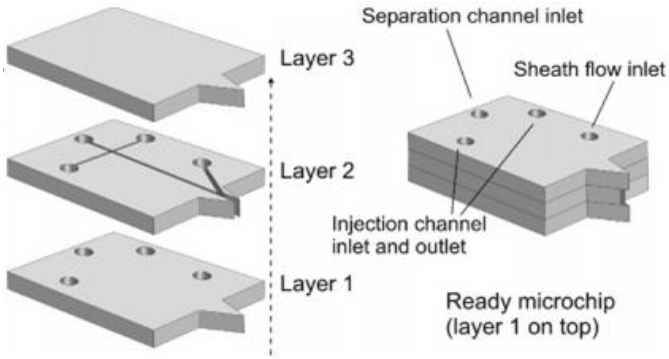


Figure 8 Schematic picture of the patterning order of the three structural SU-8 layers (left) and the prepared MCE-ESI/MS microchip released from the silicon wafer and flipped over (right).[48] *Reproduced with permission of the American Chemical Society.*

4.4 Integrated sample pretreatment

4.4.1 On-chip solid-phase extraction

A porous polymer monolith was fabricated within the injection cross of the microchip shown in Figure 7A. The methacrylate monolith was prepared according to a protocol slightly modified from that previously presented.[149,150] The monomer mixture, thoroughly described in publication IV, was loaded into the microchannel and photopolymerized with a UV laser through the SU-8 cover layer (70-µm-thick) at the injection cross for 7 min (Figure 9).

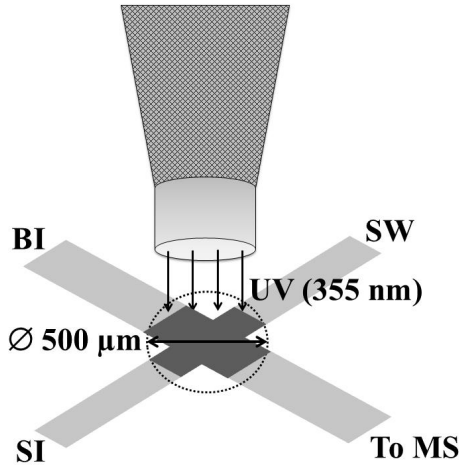


Figure 9 Schematic view of the photopolymerization setup used for preparing the monolith by beaming a UV laser at the injection cross of the SU-8 MCE-ESI/MS microchip.

Rinsing of the porogens and unpolymerized methacrylate monomers from the monolith was found to be crucial in minimizing background disturbance and suppressing ionization in ESI/MS. For this, the washing procedure was optimized with the aid of fluorescent-labeled porogens (fluorescein-PEG, 5k). Now the washing efficiency could be monitored as the weakening fluorescence of the monolith pattern, in addition to the quality of the mass spectra (Figure 10). The optimized washing procedure included rinsing the monolith with a water/methanol 1:1 solution by vacuum suction for 10 minutes before and after keeping the microchips in the same solution for one hour. This procedure was found adequate to ensure high quality MS analysis (Figure 10B). As is evident from Figure 10A, unless they are carefully removed from the monolith before use, the PEG residues will reduce the quality of the mass spectra.

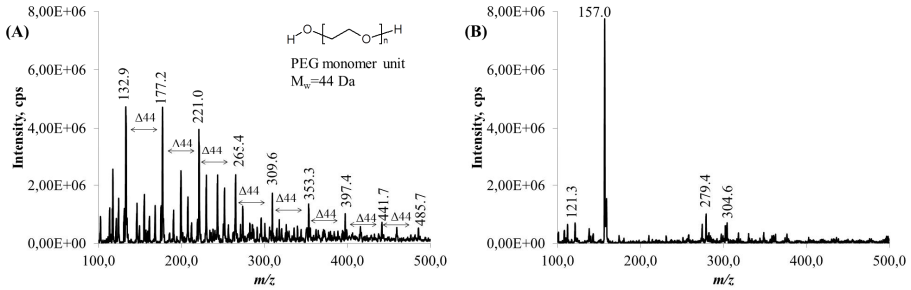


Figure 10 ESI/MS spectra of a background electrolyte (BGE) run obtained with an SU-8 MCE-ESI/MS chip with porous methacrylate pattern at the injection cross: (A) after inadequate washing, showing the characteristic PEG pattern and (B) after a one-hour water/methanol 1:1 wash (60°C) and additional rinsing by vacuum suction for 10 min, showing a negligible number of different background ions.

4.4.2 On-chip liquid-phase microextraction

On-chip LPME was performed as previously described[146] and is illustrated in Figure 11. An aliquot of 2 µL of 0.1% formic acid (pH 2.7) with 100 µM verapamil as an internal standard (acceptor solution) was applied to the sample inlet (SI) of the SU-8 MCE-ESI/MS microchip (Figure 7A). A 5x5 mm piece of Celgard 2500 microporous polypropylene membrane with 25-µm-thick, 55% porosity, and 0.21 x 0.05 µm pores was wetted with 1-octanol and placed on top of the acceptor solution. Finally, 4 µL of alkaline urine sample (30 mM sodium hydroxide, pH 11.4, donor solution) was applied on top of the membrane to initiate extraction. After 5 min the sample (donor) droplet was pipetted away, the polypropylene membrane was removed, and a platinum electrode was placed in the acceptor solution in the sample inlet (SI). The injection voltages were immediately applied and the injection was performed in pinched mode for 60 s before application of the MCE separation voltages.

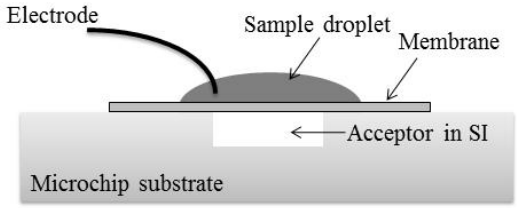
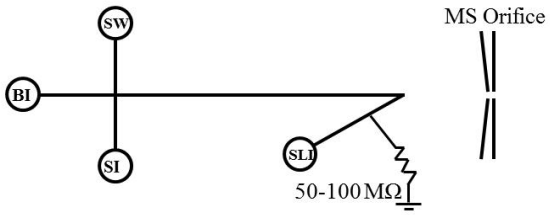


Figure 11 Schematic illustration of the on-chip LPME system on top of an SU-8 MCE-ESI/MS microchip. The sample solution was alkaline (30 mM NaOH, pH 11.4) urine. The acidic (0.1% formic acid, pH 2.7) acceptor solution contained 100 µM verapamil as internal standard.

4.5 Operation of the microchips

The microchips were placed on an *xyz*-aligning stage in front of a triple quadrupole MS (II-IV), an ion trap MS (I, II), or a quadrupole time-of-flight (Q-TOF) MS (I). The liquid reservoirs were filled with appropriate solutions as explained in the original publications I-IV. An external power supply was used for application of the injection and separation voltages through platinum wires placed in the liquid filled inlets. In MCE the sample injection was performed in pinched injection mode by applying a voltage difference between the sample inlet (SI) and the sample waste (SW), while a small focusing potential was applied to the buffer inlet (BI). During injection, the sheath liquid inlet (SLI) was floating, so that no spray was produced. The MCE separations were performed in cathodic mode with electric field strengths of 375-800 V/cm between the BI and SLI with small pushback voltages applied to the SI and SW. The effective separation length of the MCE channel was 2 or 4 cm.

In cIEF (II) the separation microchannel (Figure 7A, bilateral sheath flow interface) was first loaded with the sample solution (0.2 mg/mL of angiotensin I and II in 2% ampholyte solution pH 3-10) by capillary force. After loading, reservoirs BI and SLI were filled with catolyte (1% ammonium hydroxide) and anolyte (80:20 methanol/water with 1% acetic acid), respectively, and focusing potentials (BI grounded and SLI 3.0 kV) were applied. Focusing was performed for 60-120 s, after which the solutions in BI and SLI were changed to BGE and fresh sheath liquid, respectively. The focused analytes were then mobilized toward the MS by applying a voltage difference between the BI (3.6 kV) and the SLI (2.0 kV). The voltage in SLI simultaneously served as the electrospray voltage. A schematic view of typical voltage setups during MCE-injection, cIEF-focusing, MCE-separation, and cIEF-mobilization is shown in Figure 12.



	BI	SLI	SI	SW
Injection (MCE)	HV (pushback)	flt	HV	gnd
Focusing (cIEF)	gnd	HV	flt	flt
Separation (MCE)	HV	HV (counter/ ESI voltage)	HV (pushback)	HV (pushback)
Mobilization (cIEF)	HV	HV (counter/ ESI voltage)	flt	flt

HV, high voltage; flt, floating; gnd, grounded

Figure 12 Schematic view of the voltage setup during MCE-injection, cIEF-focusing, MCE-separation, and cIEF-mobilization followed by ESI/MS detection.

For the cIEF-transient ITP (tITP) separations (II), the cIEF channel was first loaded with the sample solution (100 µM peptides in 2% ampholyte with 0.33% TEMED) by applying a voltage difference (1.5-3.0 kV) between reservoirs SI and BGE (Figure 7B) for 100-150 s. Then, reservoirs AN and CA were filled with anolyte (1% formic acid) and catolyte (0.5% ammonium hydroxide), respectively. cIEF-tITP separations were performed in steps as follows: After loading, the cIEF focusing voltage (1.5-6.0 kV) was immediately applied between the catolyte (CA) and the anolyte (AN) solutions for 200-270 s. In a final step the focused analytes were mobilized and further separated by applying a positive voltage difference between reservoirs BGE (6 kV) and SLI (3.5 kV). The counter voltage applied to reservoir SLI simultaneously served as the electrospray voltage and was typically 2.5 kV relative to MS. In both microchip designs, the separation current (typically 20-40 µA) was divided at the sheath flow intersection so that the electrospray current was 50-300 nA and the excess current was led to ground through a 50 MΩ (ion trap and QqQ) or 100 MΩ (Q-TOF) resistor coupled in parallel with the ES voltage power supply.

5 RESULTS AND DISCUSSION

The main results of the research are shortly described in this chapter. Details can be found in the original publications I-IV.

The SU-8 microchips were first used in the identification of standard and tryptic peptides (I, II) and peptides from a human cell lysate (I), and for screening of human urine for drug metabolites (III). Peptide separations were performed by both on-chip MCE (I) and on-chip cIEF (II) and followed by online ESI/MS detection, whereas the urine samples were analyzed by MCE-ESI/MS after off-chip SPE (III).

The applicability of the SU-8 microchips to quantitative experiments was preliminarily tested. Human urine samples were precleaned and concentrated on-chip by LPME (III), and a porous polymer monolith was fabricated *in situ* at the microchip injection cross for SPE and preconcentration of drug molecules before MCE-ESI/MS (IV). In addition, Michaelis-Menten kinetics was successfully determined for a CYP450-mediated probe reaction (phase I oxidation of bufuralol to 1-hydroxybufuralol) (III).

5.1 MCE-ESI/MS in peptide analysis and proteomics (I, II)

Typically, the preparation and analysis of a proteome sample begins with cell cultivation and subsequent lysis to separate the proteins from the cell debris. For identification and quantification of the proteins in the mixture, the sample is enzymatically digested (usually with trypsin), the peptides are separated (usually chromatographically), and finally the peptides are analyzed by ESI or MALDI MS/MS. The MS/MS spectra are then studied for protein identification and quantification.[151] A LOC device potentially integrates all these operations and, additionally, can be tailored for specific analytical needs for instance, by coupling microchip LC to MS.[46,152,153] Such LC-ESI/MS microchips have also been demonstrated, and separation of tryptic peptides of bovine serum albumin (BSA) has been performed at low fmol levels.[46] However, the speed of analysis has not much exceeded that of conventional analysis systems. The expected rapidity of microchip analysis has not been achieved, and there is no significant improvement in sample throughput as compared with conventional instrumentation. Integrated MCE-ESI/MS, in turn, provides improved performance in terms of sample throughput.[60,61]

In this work, SU-8 microfabrication technology was utilized for the fabrication of microchip systems for proteomics research. The microfluidic platform (Figure 7A), comprising a sample introduction unit and an

electrophoretic separation channel with a monolithically integrated ESI emitter, as well as an auxiliary channel for the introduction of sheath liquid and internal mass calibrant, was used for peptide mass fingerprinting and identification of proteins in a human muscle cell lysate sample and for protein identification by MS/MS (I). In addition, microchip cIEF was directly coupled to ESI/MS detection via a bilateral sheath flow interface (Figure 7A) and mobilization unit (Figure 7B) (II).

5.1.1 Peptide mass fingerprinting and protein sequencing by MCE-ESI/MS(MS) (I)

Peptide mass fingerprinting followed by protein identification was performed for four proteins (cytochrome *c*, β-lactoglobulin, ovalbumin, and BSA) with molar masses ranging from 12 to 69 kDa. Typically, a number of singly or doubly charged tryptic peptides were identified, and sequence coverages between 50% and 70% were routinely obtained for all proteins (Table 7).

Table 7. *Summary of the MCE-ESI/MS analysis of the tryptic digests of cytochrome c, β-lactoglobulin, ovalbumin, and BSA.*

Protein	M_r (kDa)	*pI*	Amino acids	GRAVY[a)]	Observed peptides [b)]	Sequence coverage (%)[b)]	Probability score[b)]	Expectation value[b)]
Cytochrome *c* (CYC_BOVIN)	12	9.52	104	-0.866	10	67	87	2.0×10^{-4}
β-Lactoglobulin (LACB_BOVIN)	18	4.76	162	-0.167	17	68	97	1.8×10^{-5}
Ovalbumin (OVAL_CHICK)	43	5.20	386	-0.006	19	59	93	5.4×10^{-5}
BSA (ALBU_BOVIN)	69	5.78	583	-0.476	46	54	177	2.0×10^{-13}

[a)]Grand average of hydropathicity index, [b)]Based on Swiss-Prot database search by MASCOT online search engine.

Accurate mass measurements greatly facilitate detailed analysis and are particularly important in the analysis of intact proteins whose masses may differ by only small amounts. For example, the mass difference between the amino acid residues lysine and methionine is 2.94553 Da, which means a 245, 29, and 15 ppm difference for 12 000, 100 000, and 200 000 Da proteins, respectively. When coupling a separation microchip to e.g., a high resolution Q-TOF instrument it is necessary to replace the original ion source with a tailor-made chip stage. However, the layout of the SU-8 microchip (Figure 7A) is also convenient for the introduction of an internal mass calibrant together with the sheath liquid and thus allow as good mass accuracy for chip-based experiments as for conventional counterparts on Q-

TOF or related instruments. Here, bradykinin fragment 1-5 (m/z=573.3149) was chosen as internal mass calibrant and was applied along with the sheath liquid. In this manner, it did not interfere with the analytes of interest during separation, but enabled identical online mass calibration for all separated analytes. Figure 13 shows the extracted ion electropherograms (EIE) and mass spectra of selected peptides from the β-lactoglobulin tryptic digest analyzed by MCE-ESI coupled to a high resolution Q-TOF MS instrument. Both the EIEs and mass spectra show good signal-to-noise ratios and allow clear identification of the peptide fragments, with a typical mass error of only about 10 ppm (Figure 13).

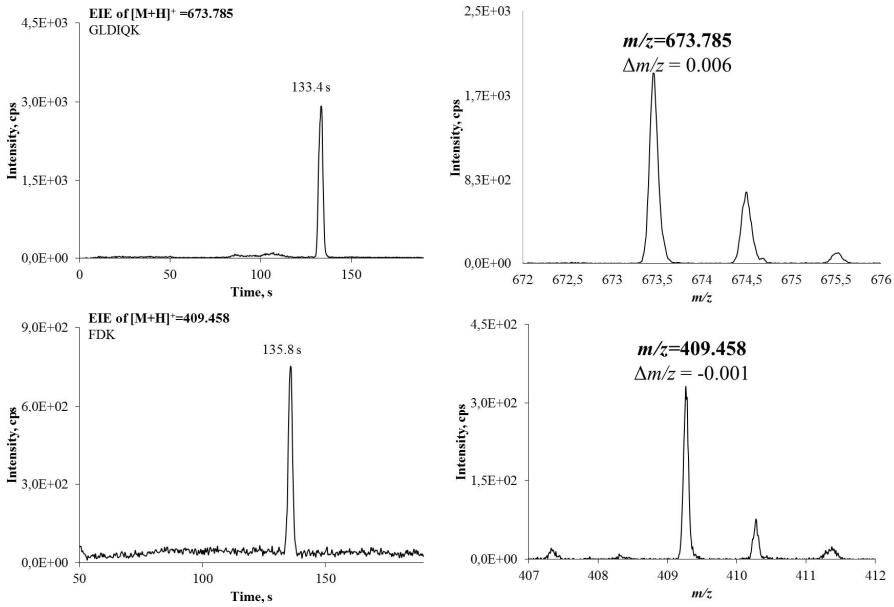


Figure 13 Selected EIEs and corresponding high resolution mass spectra from the MCE-ESI/MS analysis of β-lactoglobulin tryptic digest. The BGE was 30 mM ammonium acetate with 50% methanol and the sheath liquid was 80:20 (v/v) methanol/water with 1% acetic acid including 5 μM bradykinin fragment 1-5 as an internal mass calibrant. Electric field strengths during injection and separation were 1250 V/cm and 400 V/cm, respectively, and the effective separation length was 4 cm.

The power of MS lies not only in the accuracy of the mass measurement (ppm for TOF-MS), but in the capacity for tandem MS (MS/MS) measurements, which provide additional information specific for the peptide amino acid sequence and, ideally, protein identification based on a single peptide MS/MS spectrum. Here, MS/MS characterization of selected tryptic peptides from cytochrome *c* and β-lactoglobulin was performed in addition to peptide mass fingerprinting. The product ion spectra of the selected precursor tryptic peptides are characterized by the easily identifiable y and b

series fragment ions (Figure 14). Both cytochrome *c* and ß-lactoglobulin could be reliably identified by the Mascot® online search engine[154] on the basis of MS/MS fragmentation data of a single tryptic peptide of each, i.e., *m/z*=1456.7 for cytochrome *c* and *m/z*=934.2 for ß-lactoglobulin. The Mascot scores, indicating identity or extensive homology to the proteins in question, were 67 (expectation value 8.3×10^{-5}) for cytochrome *c* and 42 (expectation value 0.028) for ß-lactoglobulin.

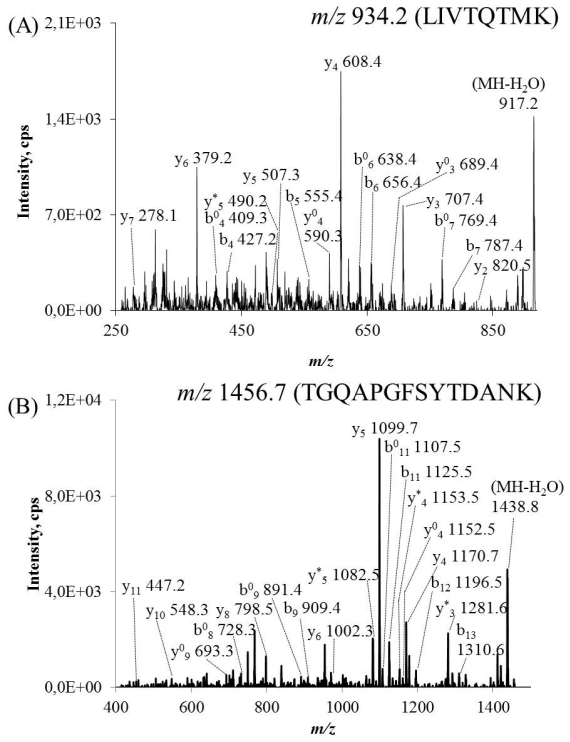


Figure 14 MS/MS spectra of selected singly-charged tryptic peptides of (A) β-lactoglobulin (*m/z*=934.2) and (B) cytochrome *c* (*m/z*=1456.7) as precursor ions from the MCE-ESI/MS/MS analysis. The BGE was 30 mM ammonium acetate with 50% methanol and the sheath liquid was 80:20 (v/v) methanol/water with 1% acetic acid. Electric field strengths during injection and separation were 1000 V/cm and 375 V/cm, respectively, the effective separation length was 4 cm, and the fragmentation amplitude was 2.0 V.

5.1.2 Identification of proteins in a human cell lysate (I)

The applicability of the SU-8 MCE-ESI/MS chip to the analysis of authentic biological samples was shown by the analysis of a tryptic digest of a human muscle cell lysate. Despite the complexity of the cellular matrix, seven proteins of different size, *pI* value, and hydrophilicity were identified in the sample (Table 8). The Mascot® probability-based scores[154] ranged from 102 to 211, indicating significant homology between detected and theoretical

tryptic peptides, and sequence coverages as good as 93% were obtained. In addition to rigorous screening against blank samples in order to eliminate potential false positives, all proteins listed in Table 8 were also identified by in-house HPLC-ESI/MS analysis of the same sample.

Table 8. *Proteins identified in digested human muscle cell lysate by MCE-ESI/MS analysis. The separation BGE was 30 mM ammonium acetate with 50% methanol and the sheath liquid was 80:20 (v/v) methanol/water with 1% acetic acid. Electric field strengths during injection and separation were 1000 V/cm and 650 V/cm, respectively, and the effective separation length was 2 cm.*

Protein	M_r, kDa	*pI*	Amino acids	GRAVY[a)]	Number of peptides[b)]	Sequence coverage, %
Q70VV1; rhesus blood group D antigen	5.6	6.51	48	0.085	5	85
Q6PCD6; GLS2 protein	6.6	11.35	61	-1.075	8	93
Q8N9L7; CDNA FLJ36925 fis, clone BRACE2005169	13.5	9.8	120	-0.756	8	70
CAF00094; DNAJ (Hsp40) homolog, subfamily C member 11	14.4	5.22	119	-1.071	10	65
Q96HG5; actin beta, (fragment)	41.0	5.56	368	-0.202	17	48
JC4775; interferon induced double-stranded RNA activated protein	57.5	5.83	504	-0.679	23	32
Q6PIC7; MAP7 domain-containing protein	74.1	8.68	660	-1.115	21	19

[a)] Grand average of hydropathicity index. [b)] Based on SwissProt database search by Mascot online search engine.

5.1.3 Interfacing of microchip cIEF with on-chip ESI/MS for peptide analysis (II)

cIEF is an established technique based on separation of zwitterionic compounds (e.g., peptides and proteins) according to their *pI* values and followed by electrokinetic or hydrodynamic mobilization of the focused pH gradient toward the detector.[63] Microchip cIEF has also been implemented for rapid separations of proteins, though usually in combination with linear

(optical) imaging of the cIEF gradient through the cover layer, without the mobilization step.[68,70,72] The coupling to MS detection is rare because the carrier ampholytes required for successful cIEF tend to suppress the ionization efficiency of the target compounds.[73,74] As a result, the potential of microchip cIEF-MS in proteomics research is often overlooked. Here, two different chip designs were introduced to facilitate the online coupling of cIEF to on-chip ESI/MS detection. The first design (Figure 7A) was used for direct EOF-driven mobilization (of peptides focused by cIEF) to the MS via an integrated (two-sided) sheath flow interface. The second design (Figure 7B) incorporated two subsequent, intersecting separation channels where peptide focusing by cIEF was performed in the first dimension and then tITP separation during mobilization through the second dimension. Also in this case, an integrated (one-sided) ESI sheath flow interface was used.

5.1.3.1 Direct coupling of microchip cIEF to MS via an on-chip sheath flow interface

The feasibility of direct coupling of microchip cIEF to ESI/MS detection was examined by using a separation microchip with a bilateral on-chip sheath flow interface. With the symmetrical interface (Figure 7A) sheath liquid could be fed to the ESI tip from both sides of the separation channel. The organic sheath flow enhances the ESI process, especially when running buffers/samples contain high amounts of water. Here the sheath liquid also minimized major background interferences due to the carrier ampholytes and as a result, the carrier ampholytes were not observed when the cIEF pattern was mobilized to the MS (Figure 15). The acidic sheath liquid also served as the anolyte during cIEF so that the pH gradient (pH 3-10), along with the focused peptides, could be directly run to the MS after focusing. Microchip cIEF-ESI/MS was demonstrated with the model compounds, angiotensin I (*pI* 7.70) and angiotensin II (*pI* 7.54).[155] The two peptides, having very similar *pI* values, migrated at about 23 s (Figure 15). Although the two peptides did not separate from each other because of their similar *pI* values, they migrated as sharp, focused bands with peak capacity of about 86, indicating that efficient focusing takes place during the cIEF step.

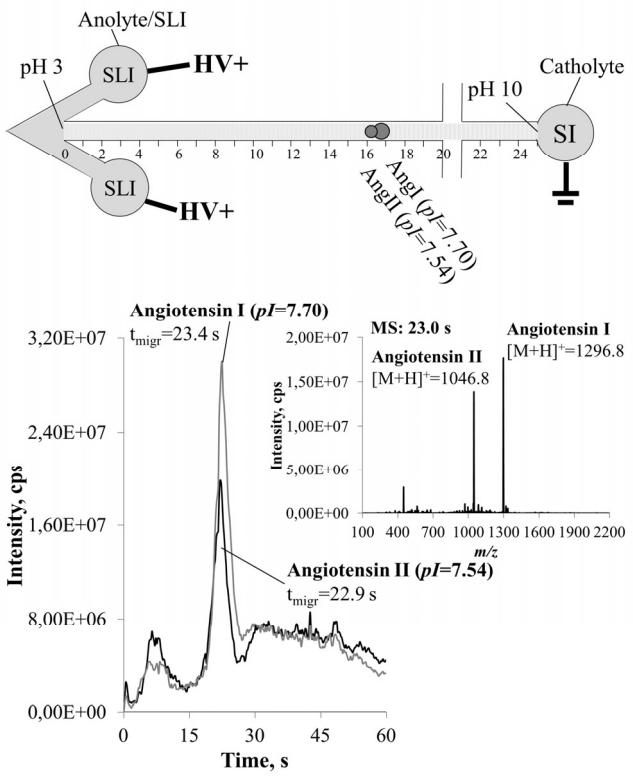


Figure 15 Schematic illustration of the theoretical position of angiotensin I and II after cIEF separation and extracted ion electropherograms (EIE) of the same peptides (both 0.2 mg/mL) following cIEF separation, mobilization, and ESI/MS detection. The size of the circles in the schematic illustration assimilates the molar masses of the peptides. Time t=0 s in the EIE corresponds to the end of the focusing step and start of the mobilization step. The focusing was performed in 2% ampholyte solution (pH 3-10) including 25% methanol at 2400 V/cm for 60 s. The mobilization was done by feeding BGE (20 mM ammonium acetate with 25% methanol) from the SI at a field strength of 800 V/cm. The mass spectrum recorded at 23 s (insert on the right, no background substraction) shows abundant protonated ions of angiotensins I and II and negligible background interference due to the carrier ampholytes.

This setup is somewhat similar to that described by Wen et al. (Figure 5)[75] except that, in this work, actuation of the cIEF chip was based purely on electrokinetic flow instead of the pressure-driven flow used previously. This was possible owing to the fact that the microchip was made of SU-8. Namely, the surface charge of SU-8 is inherently pH-dependent; basic and acidic buffers maintain cathodic and anodic EOF, respectively.[156] Thus, the flow direction close to the anode (low pH) is opposing that at the cathode (high pH) so that the net velocity in the SU-8-based channel during cIEF approaches zero. With most other microchip materials the cIEF separation channels need to be coated in order to eliminate or significantly reduce the EOF to allow for complete focusing of the analytes.[67] The advantage of having uncoated (charged) surfaces arise from the fact that, after focusing,

EOF can be used for mobilization instead of pressure-driven flow. Thanks to the relatively short separation channel, the cIEF focusing time in this work was also significantly shorter (60 s) compared to that applied (~10 min) in the previous work in which a 16-cm-long cIEF channel was used.[75]

5.1.3.2 Coupling of cIEF to MS via a transient-ITP unit

In addition to direct coupling of cIEF to ESI/MS another microchip design, with multiple intersecting channels for isolation of the cIEF separation unit from the ESI interface, was also developed (Figure 7B). This design permitted cIEF separation in the first part of the channel, fully isolated from the ESI interface by a second separation channel for tITP. Before operation, the separation microchannels and the sheath liquid channel were filled with BGE and sheath liquid, respectively, by capillary force. After this the operation of the microchip was monitored via electrical currents during electrokinetic loading (with 2% ampholyte solution), focusing, and mobilization (Figure 16). Some interesting observations were made. As can be seen from Figure 16, the total amount of charges decrease during the first 25 s when the cIEF channel is filled with ampholyte solution. Simultaneously the pH gradient is formed by the carrier ampholytes in the sample plug entering the cIEF channel. As a result, the sample components and the carrier ampholytes start to arrange according to their *pI* values already during the loading step. However, a subsequent increase in current occurs between 45 and 55 s (Figure 16), suggesting progressive migration of the acetate counterions (from the BI into the cIEF channel) and thus drift in the pH gradient and slow fluctuation of the electrical current between 50 and 100 s (Figure 16), similar to earlier work with conventional CE capillaries.[157] The sample components (or the carrier ampholytes) do not readily reach their isoelectric points during the loading step. Thus, the cIEF focusing must be finalized during the second cIEF step (Figure 16) by switching to potential difference between the anolyte (AN) and the catolyte (CA). However, due to the arrangement of species during the beginning of the loading step, a relatively short focusing time is required here. The current drop during the focusing step (Figure 16) indicates the formation of a more stable pH gradient, within about 60 s, with the steady-state current leveling off at about 15 μA (8.9% RSD, n=6 repeated runs).

Finally, the pH gradient is electrokinetically mobilized toward the MS (Figure 16). However, the pH gradient is preceded by a relatively long plug of the running buffer which acts as a leading electrolyte (LE). The ampholyte solution also acts as the terminating electrolyte (TE) and instead of just mobilizing a cIEF gradient, the separation mode is more likely tITP as described.[157] This means that the observed migration order is affected by both *pI*- and μ_{EP} values of the sample components. The *pI* mainly determines the position of the sample component in the cIEF channel, and thus its (individual) effective separation length from the cIEF channel to the ESI tip.

The intrinsic μ_{EP}, in turn, determine the speed at which each sample component is migrating toward the MS. This is illustrated with the separation of histidine (*pI* 7.55)[155] and angiotensin I (*pI* 7.70)[155] in Figure 16. Despite the fact that the *pI* values are very close to each other (Δ*pI* 0.15), these two compounds are baseline-resolved (RS=1.9) thanks to their very different size and thus, different intrinsic μ_{EP} values.

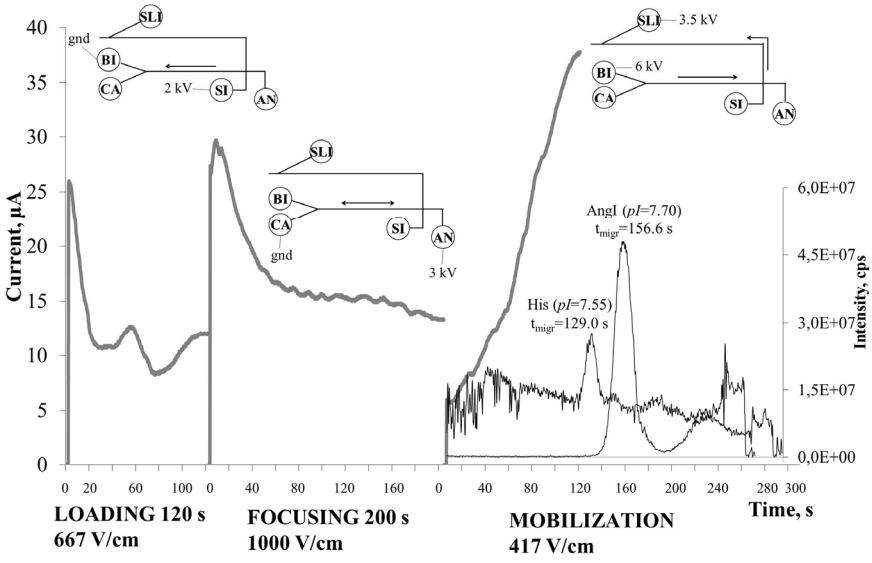


Figure 16 The electrical current profiles (average of six repeated runs) during sample loading (SI→BI), focusing (AN→CA), and mobilization (BI→SLI) along with the EIEs of histidine ($[M+H]^+$=156.0) and angiotensin I ($[M+2H]^{2+}$=649.8) separated by cIEF-tITP-ESI/MS. The anolyte (AN) was 1% acetic acid, the catolyte (CA) 1% ammonium hydroxide, the BGE (BI) 30 mM ammonium acetate with 30% methanol, and the sheath liquid (SLI) methanol:water 80:20 (v/v) with 1% acetic acid.

Figure 17 shows the use of this setup in the separation of a mixture of five peptides (bradykinin (fragment 1-5, *pI* 10.55), angiotensin III (*pI* 9.35), substance P (fragment 6-11, *pI* 7.81), angiotensin I (*pI* 7.70), and angiotensin II (*pI* 7.54)),[155] with different *pI* values. The most basic peptides were prevented from focusing in a distal region of the microchannel (i.e., beyond the Y intersection close to inlet CA) by adding 0.33% (v/v) TEMED to the ampholyte solution[158,159] and thereby shift the theoretical position of pH 10 3.3 mm downstream from the Y intersection (Figure 17). Under these conditions, the most basic peptide, bradykinin (*pI* 10.55), migrates and focuses to the interface of TEMED and the ampholyte solution (pH 10) after which it is diffused into the TEMED zone (Figure 17). The theoretical position of the other peptides after the focusing step is given according to their *pI* values.

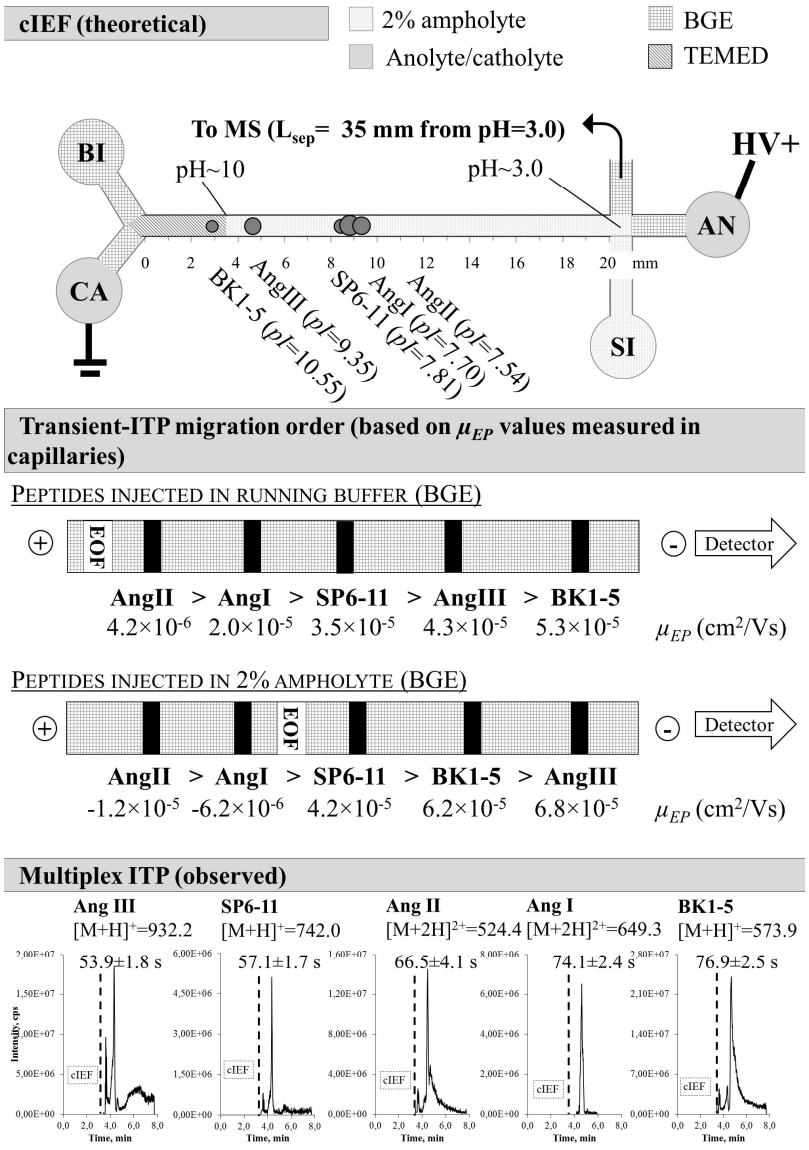


Figure 17 Schematic illustration of the theoretical positions of bradykinin fragment 1-5 (BK1-5), angiotensin III (AngIII), substance P fragment 6-11 (SP6-11), angiotensin I (AngI), and angiotensin II (AngII) following cIEF. The size of the circles assimilates the molar masses of the peptides. The theoretical ITP migration order is given based on the intrinsic electrophoretic mobilities (μ_{EP}) measured in conventional silica capillaries by injecting peptides in the running buffer or in the 2% ampholyte solution. The EIEs of the same peptides from cIEF-tITP analysis show both the focusing (200 s at 1333 V/cm) and mobilization (417 V/cm) steps. Loading of the cIEF channel with the sample solution was performed at 1000 V/cm for 100 s. The sample solution contained peptides (each 100 μM), 2% ampholyte (pH 3-10), and 0.33% TEMED. The anolyte was 1% formic acid, the catolyte was 0.5% ammonium hydroxide, the BGE was 30 mM ammonium acetate with 30% methanol, and the sheath liquid was methanol:water 80:20 (v/v) with 1% acetic acid. Time t=0 s in the electropherograms corresponds to the end of the focusing step (also indicated with dashed line).

As is evident from Figure 17, the migration order of the peptides does not follow pure cIEF, but is largely affected by their intrinsic μ_{EP} values. This is particularly evident for angiotensin III and substance P fragment 6-11, both of which show relatively high cathodic mobilities and thus overtake angiotensins I and II (featuring slow anodic mobilities in the ampholyte solutions) during the mobilization step (Figure 17). Instead, bradykinin, which also shows high cathodic mobility, but is trapped in the TEMED zone based on its *pI* value, will not catch the angiotensins I and II and migrates the last. Comparison of the peptides' intrinsic μ_{EP}:s in the plain BGE and in the 2% ampholyte solution also reveals that addition of ampholytes into the running buffer has a clear impact. This is particularly the case of angiotensins I and II, which shift from cathodic to anodic mobility. As a result, three peptides with nearly identical *pI* values (i.e., angiotensin II, *pI* 7.54; angiotensin I, *pI* 7.70; substance P, fragment 6-11, *pI* 7.81[155]) are efficiently resolved in cIEF-tITP mode (Figure 17). The repeatability of the migration times (after focusing and mobilization) was also relatively good (3.1-6.8% RSD, n=3).

5.2 MCE-ESI/MS in metabolomics research (III)

The development of potential drug candidates frequently is terminated when adsorption, distribution, metabolism, and excretion (ADME) properties are found to be poor. Expensive terminations in late clinical stages could be avoided if these properties were clarified much earlier in the drug discovery process.[160] Metabolic profiling of new drug candidates is particularly important and includes not only the identification of metabolites but also screening of their properties.[161] Equally important are screening of drug–drug interactions and determining the kinetic parameters of the drug metabolism. In these types of analyses, the key issue is high throughput, i.e., analyzing as many samples as possible in a short time.

Here, the applicability of the microchips to metabolics research was demonstrated by screening for metabolites of tramadol and paracetamol in authentic human urine samples and by determining the kinetic parameters of the CYP450 mediated bufuralol 1-hydroxylation.

5.2.1 Metabolic profiling

The MCE-ESI/MS microchips were used for metabolic profiling of human urine samples after off-chip SPE. As presented in Figure 18, six tramadol metabolites, including phase I and phase II products, as well as tramadol itself were detected and separated from each other by MCE-ESI/MS.

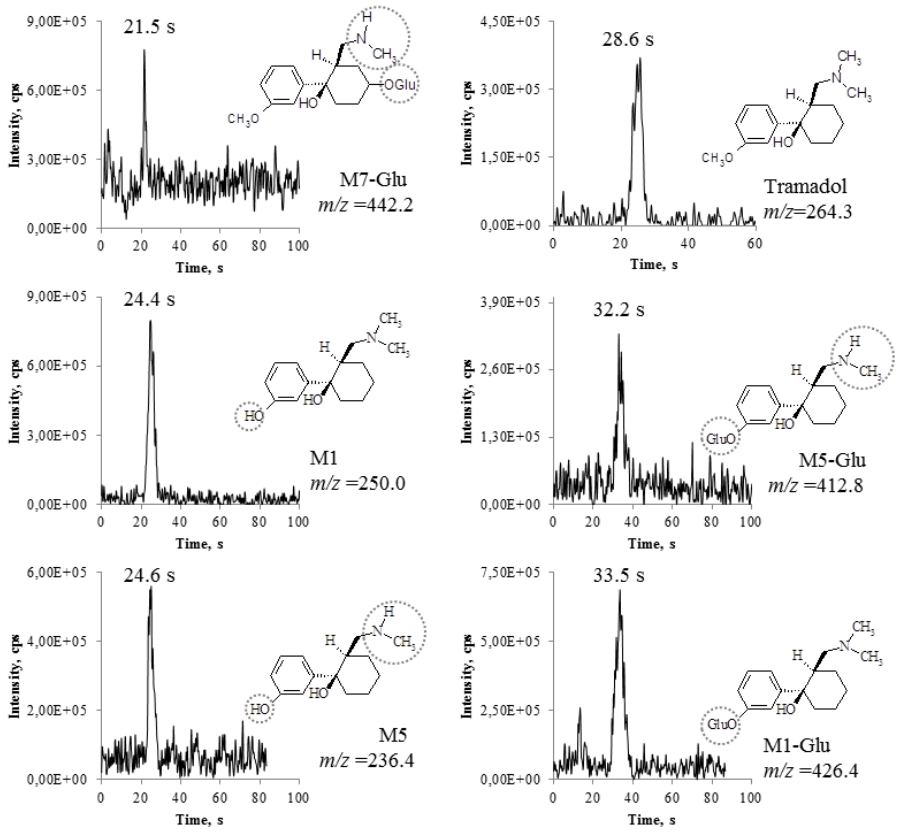


Figure 18 EIEs of tramadol and its metabolites detected in a human urine sample and separated by MCE-ESI/MS after off-chip SPE (5-fold preconcentration). The BGE was 30 mM ammonium acetate with 50% methanol and the sheath liquid was 80:20 (v/v) methanol/water with 1% acetic acid. The electric field strengths during injection (20 s) and separation were 1000 and 800 V/cm, respectively. M1, *O*-desmethyltramadol; M5, *N,O*-didesmethyltramadol; M1-Glu, glucuronide of *O*-desmethyltramadol; M5-Glu, glucuronide of *N,O*-didesmethyltramadol; M7-Glu, glucuronide of 4-hydroxy-ciclohexyl-*N*-desmethyl tramadol.

In the case of paracetamol (Figure 19), mostly phase II conjugation products (glucuronide, glutathione, and cysteine) were detected, in addition to paracetamol itself. With use of tramadol and paracetamol as reference compounds, the feasibility of the MCE-ESI/MS system could be tested for a broad range of metabolites with diverse chemical and physical properties. According to the literature, tramadol is extensively converted to several phase I oxidation products,[162,163] while the main urinary metabolites of paracetamol are phase II conjugation products, such as glucuronides.[164,165] Thus, the metabolite findings for both tramadol (Figure 18) and paracetamol (Figure 19) are in good accordance with previous results.

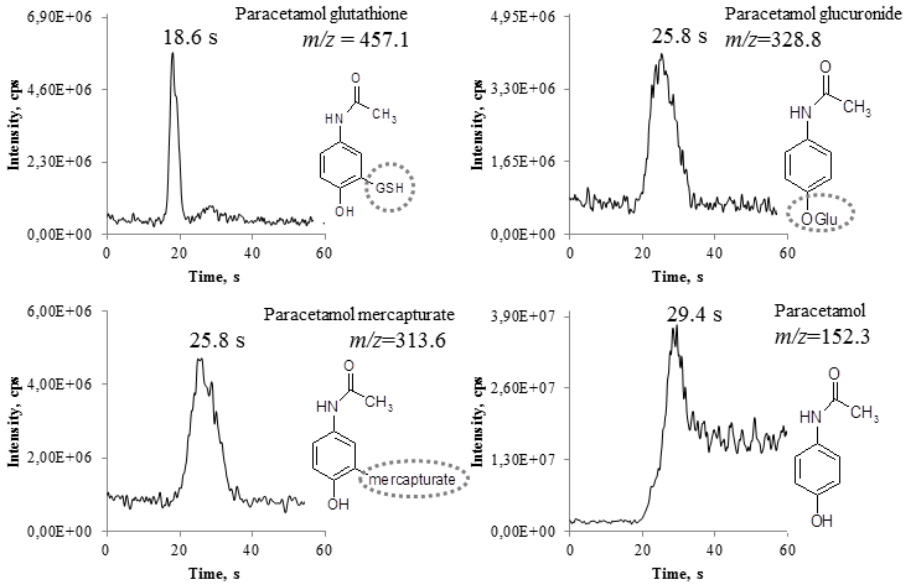


Figure 19 EIEs of paracetamol and its metabolites separated from a human urine sample by MCE (effective separation length 2 cm) and detected by ESI/MS after off-chip SPE (5-fold preconcentration). The BGE was 30 mM ammonium acetate with 50% methanol and the sheath liquid was 80:20 (v/v) methanol/water with 1% acetic acid. The electric field strengths during injection (20 s) and separation were 1000 and 500 V/cm, respectively.

5.2.2 Enzyme kinetics

Enzyme kinetics is a discipline that focuses on the reaction rates of enzymatically catalyzed chemical reactions. In a typical enzyme-catalyzed reaction the substrate is bound to the enzyme, converted to a product and released. The Michaelis-Menten constant (K_m) describes the substrate concentration at which the reaction velocity is half of the maximal reaction rate (V_{max}). In this work, the kinetic parameters (K_m and V_{max}) for bufuralol 1-hydroxylation in human liver microsomes (HLM) were determined to demonstrate the applicability of the SU-8 microchips to quantitative enzyme kinetics studies. The CYP450-mediated metabolism was shown to follow Michaelis-Menten kinetics with K_m and V_{max} values of 55 µM and 147 pmol/min/mg protein, respectively (Figure 20). These kinetic parameters compare well with the values determined by in-house HPLC-UV (K_m= 31 µM, V_{max}= 185 pmol/min/mg protein) as well as with literature values (K_m 50–250 µM[166] and V_{max} 60–240 pmol/min/mg protein).[167]

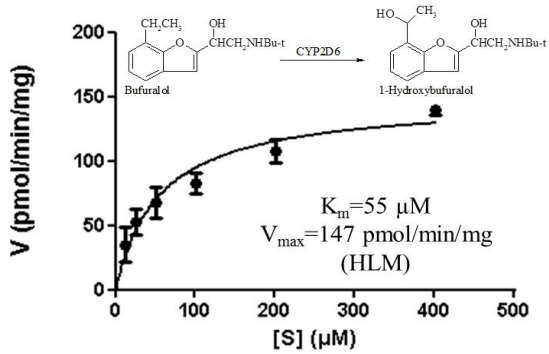


Figure 20 Michaelis-Menten kinetics of the CYP450-mediated conversion of bufuralol to 1-hydroxybufuralol in human liver microsomes (HLM) determined by MCE-ESI/MS in selected reaction monitoring (SRM) mode. The separation was performed under electric field strength of 750 V/cm in 30 mM ammonium acetate with 50% methanol as BGE and 80:20 (v/v) methanol/water with 1% acetic acid as sheath liquid. The substrate concentration ranged from 12.5 to 400 μM and all incubations were done in duplicate at each substrate concentration.

An obvious advantage of performing MCE-ESI/MS analyses as compared to traditional HPLC-UV is the speed of analysis. Here the migration times of 1-hydroxybufuralol and bufuralol were 24.6 s and 27.0 s, respectively with the MCE-ESI/MS method as compared to 5.1 min and 6.6 min required in the in-house HPLC-UV method. In addition, the sample consumption in MCE is much less than that in HPLC. First of all, in MCE the volume pipetted to the sample inlet is approximately 3 μL (as compared to 20 μL injected in HPLC). In addition, as a result of miniaturization, multiple repeated analyses can be performed out of the 3 μL sample volume, since only a few tens of picoliter is effectively used (here 75 pL, determined by the volume at the intersection of the injection and separation channels).

5.3 On-chip sample pretreatment and preconcentration (III, IV)

As stated in the previous chapter, very small amounts of sample is required in order to perform multiple repeated analyses on a microchip platform. However, in order to improve the efficiency of sample consumption (injected vs. total sample volume required) and to reach better concentration sensitivity there is a need for coupling online sample pretreatment as an integral part of separation microchips.[96] In this work, sample pretreatment by SPE (IV) and LPME (III) was integrated directly on the SU-8 MCE-ESI/MS microchips for preconcentration of drug standards (IV) and clean-up and analysis of authentic urine samples (III).

5.3.1 On-chip preconcentration by solid-phase extraction (IV)

SPE is one of the most useful techniques for sample purification and preconcentration. In this study, a porous methacrylate monolith was integrated in the injection cross of an SU-8-based MCE-ESI/MS microchip to perform on-chip sample precleaning and preconcentration during the injection step before MCE-ESI/MS. The use of a high-power laser allowed for maskless photopolymerization of a precisely defined, cross shaped porous polymer zone *in situ* at the injection cross of the SU-8 separation chip (Figure 7A and Figure 9). In addition, the high intensity of the laser beam (15 μJ at 1 kHz, 355 nm) enabled very fast curing (here, best performance at 7 min) and initiation of the photopolymerization reaction even through a 70 μm-thick SU-8 cover layer, which itself absorbs the near UV light to some degree.[168] The layout of the injection cross also allowed firm anchoring of the monolith based on its shape and no prefunctionalization of the SU-8 surface was needed. With a view to performing rapid online preconcentration, the porosity of the monolith was compromised with relatively high through-flow (i.e., large pores for low back pressure) over flawless retention in order to allow for fully electrokinetic actuation and thus avoid the need for coupling to external pumps. The operation principle of the SPE-MCE-ESI microchip is presented in Figure 21 and illustrated with a four step protocol: (1) sample loading (in 2% ammonium hydroxide, pH 12), (2) rinsing (of unretained, hydrophilic analytes), (3) injection (in BGE, release of retained hydrophobic analytes), and (4) elution followed by simultaneous MCE separation (of released hydrophobic analytes).

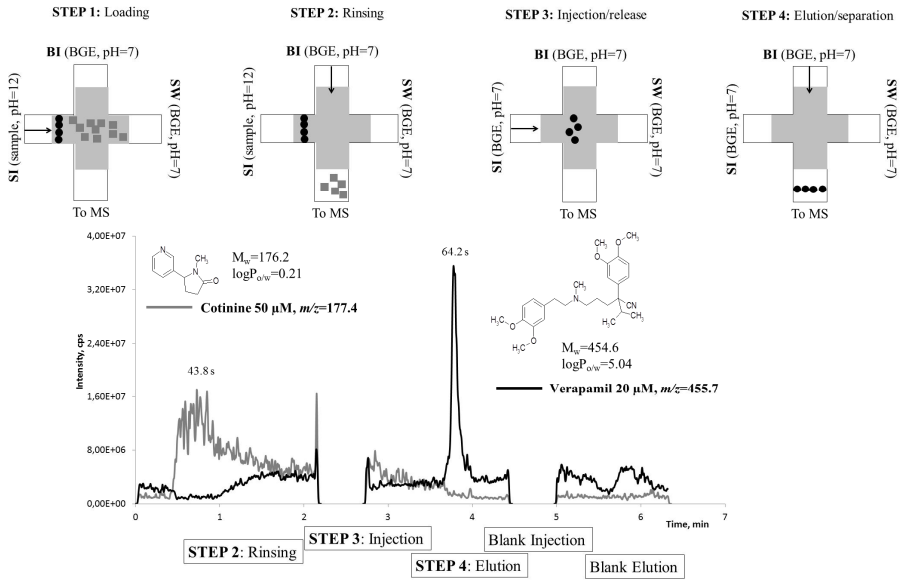


Figure 21 The operation principle of the SPE-MCE-ESI microchip is illustrated with squares (grey) and circles (black) representing hydrophilic and hydrophobic sample components, respectively. EIE of the $[M+H]^+$ ions of cotinine (grey line, 50 µM) and verapamil (black line, 20 µM) show that the hydrophilic, unretained cotinine is washed away in step 2, whereas hydrophobic verapamil is selectively retained (steps 1–2), released (step 3), and eluted to MS (step 4). The blank injection (~4.5–5 min) and elution (~5–6.5 min) in the end of the electropherogram show no memory effect, that is, all sample components are efficiently released from the monolith in steps 3–4. The sample components were loaded in 2% ammonium hydroxide solution (pH 12, 1000 V/cm, step 1) and injected in BGE (30 mM ammonium acetate with 50% methanol, 1000 V/cm, step 3). Rinsing and elution was performed in BGE (800 V/cm, step 2 and 3).

During sample loading (30 s), the hydrophilic compound cotinine ($logP_{o/w}$=0.21) was not adsorbed on the porous monolith, but rather spread throughout the monolith and gave a broad and tailing peak when rinsed to the MS (step 2). Instead, verapamil, which is more hydrophobic ($logP_{o/w}$=5.04) was concentrated at the monolith border (step 1) and released only after elution with organic solvent (50% methanol with 30 mM ammonium acetate, step 3 and 4). The repeatabilities of the migration time (RSD=3.1%, n=3) and peak height (RSD=11.5%, n=3) of the preconcentrated verapamil indicate robust performance from run to run. These values are comparable to those previously reported for similar MCE-ESI/MS analyses without SPE concentration in this work (III) and in the literature,[48,125,169] which clearly indicates that addition of on-chip SPE did not significantly interfere with the MCE separation. Furthermore, correlation was established between the loading time (10–25 s) and the peak area obtained using two test compounds with somewhat similar hydrophobicity, namely tramadol with $logP_{o/w}$=2.45 and propranolol with $logP_{o/w}$=2.58. For both compounds, the enrichment factors were directly proportional to the loading times and were

calculated to be approximately 15-fold (R^2=0.950, tramadol) and 23-fold (R^2=0.999, propranolol) at a loading time of just 25 s (Figure 22A). The availability of such short loading times (comparable to normal MCE injection times) means that our chip design enables efficient SPE without compromising the inherent high speed of MCE as illustrated with rapid SPE-MCE-ESI/MS analysis of preconcentrated pharmaceuticals in Figure 22B. With our SPE-MCE-ESI chip, the total analysis cycle time was typically less than four minutes (including loading, rinsing, elution/injection, and MCE-ESI/MS).

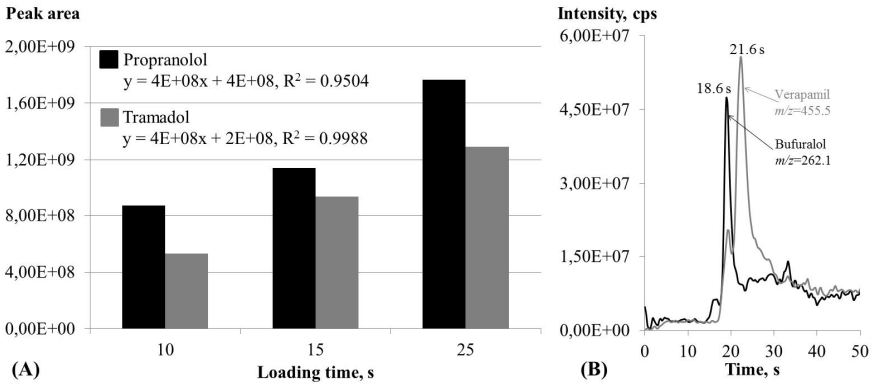


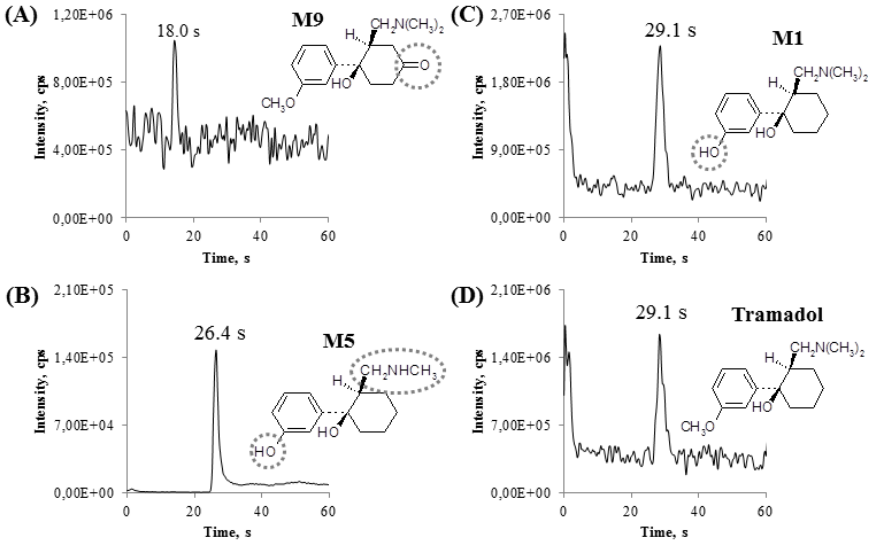
Figure 22 (A) Effect of the sample loading time (step 1) on the area of the released peaks as illustrated with propranolol ($logP_{o/w}$ = 2.58) and tramadol ($logP_{o/w}$ = 2.45). (B) Separation of preconcentrated bufuralol ($logP_{o/w}$ = 2.99; black line) and verapamil ($logP_{o/w}$ = 5.04; grey line) after simultaneous loading into the monolith (step 1, 15 s), release (step 3), and separation by MCE (step 4). Sample loading (each 25 µM) and rinsing (steps 1–3) was according to Figure 21, but the electric field strength during separation (step 4) was 900 V/cm.

5.3.2 On-chip clean-up by liquid-phase microextraction (III)

In LPME, target analytes are extracted from a biological or other matrix (pH adjusted to obtain analytes in neutral form), through a hollow fiber wetted with an organic solvent, into a suitable acceptor solution (pH adjusted to give analytes in ionic form).[137] The acceptor solution is then injected into a chromatographic or electrophoretic separation system. LPME is easily downscaled to low µL volumes by replacing the hollow fiber with a flat polypropylene membrane, which also facilitates its implementation to LOC systems.[146]

The experimental setup used in this work is shown in Figure 11. Here, the pH gradient between the donor (pH 11.4) and acceptor (pH 2.7) solutions served as the only driving force for extraction since no stirring was applied to promote mass transfer in the system. Figure 23 lists the metabolites of tramadol in addition to tramadol itself detected with the LPME-MCE-ESI/MS(MS) setup. All compounds except *N,O*-didesmethyltramadol (M5)

were easily detected even in full-scan MS mode. In contrast, metabolite M5 was only observed with the more specific and sensitive detection in SRM mode, probably because of its poorer extraction efficiency (i.e., zwitterionic nature and relatively low logD value at pH 11), which reduces its theoretical mass transfer from the donor solution to the octanol-wetted membrane.



Drug/metabolite		*m/z*	MS mode*	pK_a		logD	
				Amine	Phenol	pH 11	pH 3
(A)	M9	278.6	EIE	9.28	-	1.26	-2.23
(B)	M5	236.1→44.2	SRM	10.02	9.22	0.34	-1.32
(C)	M1	250.5	EIE	8.97	9.62	0.74	-1.20
(D)	tramadol	264.3	EIE	9.23	-	2.44	-1.05

*EIE=extracted ion electropherogram, SRM=selected reaction monitoring

Figure 23 EIEs (M9, M1, and tramadol) and SRM electropherogram (M5) of tramadol and its metabolites separated from a human urine sample by MCE and detected by ESI/MS(/MS) after on-chip LPME (2-fold preconcentration). The LPME was performed from alkaline urine (NaOH 30 mM, pH 11.4) into acidic acceptor solution (0.1% formic acid, pH 2.7) for 5 min, then injected (60 s, 1000 V/cm) and separated (750 V/cm) in 30 mM ammonium formate with 50% methanol. The sheath liquid consisted of 80:20 (v/v) methanol/water with 1% formic acid. Log*D* and pK_a values were derived from the online MarvinSketch chemical editor at www.chemaxon.com. M1, *O*-desmethyltramadol; M5, *N,O*-didesmethyltramadol; M9, 4-oxocyclohexyltramadol.

5.4 Summary and Critical Review of the Results

In this work, microchip based tools for various bioanalytical tasks were developed and tested. The microchips, made of SU-8 epoxy polymer, were used in the analysis of proteins and peptides (I, II) and metabolites in human urine samples (III). In addition sample pretreatment (preconcentration) steps (III, IV) were integrated into the microchips and exploited in the concentration and precleaning of drug molecules prior to online separation and detection. The polymer microchips incorporated separation channel(s), a sheath flow interface, and a monolithically integrated ESI emitter for online MS detection. Separations were performed by MCE (I, III, IV), cIEF (II), and cIEF-tITP (II) with detection by ESI/MS or MS/MS. Integration of sample pretreatment (preconcentration) was achieved by LPME (III) and SPE (IV).

The SU-8 microchips performed well in the MCE-ESI/MS analysis of tryptic peptides (I) and metabolites of tramadol and paracetamol in human urine (III). The experiments demonstrated the possibility of analyzing both proteomics and metabolomics samples fast and efficiently. A full validation of the performance of the MCE-ESI/MS microchips was not undertaken here. However, the reproducibility of the chip fabrication (batch-to-batch) has been reported,[170] and the repeatabilities in this work and previously[170] have routinely been below 5% for the migration time and below 11% for the peak area.

A great advantage of CE, and of its counterpart MCE in particular, is the very narrow peak widths, which often indicate good separation efficiency. Here, the short injection plug (determined by the intersection of the separation and injection channels) resulted in peak widths as narrow as 0.6 s at half height and plate numbers approaching 10^6 for tryptic peptides (I). In addition, the straightforward integration of the separation channel with the ESI emitter (through a monolithically integrated sheath flow interface) provided dead-volume-free junctions and negligible peak broadening. Furthermore, the sheath liquid is also advantageous if the sample contains high amounts of ES signal suppressing agents. This was shown for cIEF separation of peptides in carrier ampholyte solution (II). The carrier ampholytes easily suppress the ionization of the sample compounds, but here, due to the organic sheath liquid, carrier ampholytes were not observed in the mass spectra of a cIEF separation of angiotensins I and II (II).

Even though it is often claimed that the very small injection volumes in MCE is a great advantage it also sets limitations especially if quantitative experiments are to be performed. Namely, if quantitative analyses are to be reliable, the repeatability of the injection volume needs to be very good. With such small volumes as those characteristic of MCE, the effect of hydrostatic pressure between the open-to-air microchannel inlets becomes critical. The pressure difference induces hydrodynamic flow in the channels (in addition to the electrokinetic flow) and consequently, variations in the actual injected sample volume between injections. However, the experiments on Michaelis-

Menten kinetics for the CYP450-mediated conversion of bufuralol to 1-hydroxybufuralol (III) suggested the possibility of applying the developed method for quantitative purposes as well. The successful and reliable use of the microchips for quantitative purposes would nevertheless require a more automated setup, a rigorous microchip validation and, most importantly, an optimization of the sample injection performance.

Besides analysis, sample pretreatment (and preconcentration) by LPME (III) and SPE (IV) was integrated on-chip. LPME was found suitable for precleaning and preconcentration of urine samples before MCE-ESI/MS and was relatively easy to integrate on chip. In particular, LPME offered selectivity in the analysis of less polar phase I metabolites, which tend to be hard to detect in SPE if the conditions are optimized for the extraction of highly polar phase II metabolites. In this work, a relatively low preconcentration factor of two was applied. The low value was due to the volume of the on-chip inlets (~2 μL), but depending on the applied chip material and the fabrication method, the depth of the sample inlet could easily be reduced to a few tens of micrometers, which would correspond to acceptor volumes in the nL range. Thus, multifold sample preconcentration could be achieved with on-chip LPME in the same way as with off-chip LLE or SPE.

For on-chip SPE, a porous methacrylate monolith was prepared by laser-induced photopolymerization at the intersection of the injection and separation microchannels of an enclosed SU-8 microchannel (IV). Sample pretreatment (preconcentration) of selected drug molecules was performed during the injection step and followed by MCE separation and ESI/MS detection. Although similar on-chip SPE systems have been reported earlier (Table 3), the setup described here provides several unique features. The use of high power laser instead of the more common UV lamps enables fast curing (best performance here at 7 min) and initiation of the photopolymerization reaction through the 70-μm-thick SU-8 structures. When using UV lamps, relatively long exposure times (typically 20 to 40 min) and UV-transparent substrates are usually required. In addition, the high intensity of the laser beam allows precise definition of the UV irradiation and thus polymerization of a relatively small area (here, Ø~500 μm) with no need for masking. Thus, laser exposure significantly reduces the complexity of the monolith fabrication, while providing sufficient structural uniformity for SPE applications. What also distinguishes the present from previous designs exploiting electrokinetic flow[107,116,118,119,122] is that, here, the SPE material was shape anchored at the injection cross of a standard MCE chip so that sample loading and elution were part of the injection and separation steps, respectively. This, most of all, allowed very fast SPE, so that the rapidity of analysis characteristic of MCE was not sacrificed. A loading time of just 25 s, a time-scale comparable to a normal microchip injection time, resulted in up to 23-fold enrichment for the test compounds. To our knowledge, this loading/SPE time is by far the fastest reported for an on-chip

SPE-MCE-ESI/MS analysis (see also Table 3). A further application might also be to immobilize trypsin into the monolith and perform online digestion of proteins during the injection step. The on-chip integration of sample pretreatment (preconcentration) demonstrated that this normally time-consuming step can be performed with significant speed (III, IV).

6 CONCLUSIONS

Chip-based separation devices are gaining more and more interest as analytical tools. Microfluidics is superior to conventional instrumentation when it comes to speed, high throughput, reduced sample consumption, and the integration of multiple analytical functions with minimal or zero dead volume between elements. Recent years have seen great advances in LOC technology, such as separations with MS detection in seconds, multidimensional analysis, and devices integrating chemical synthesis and analysis. Although most publications thus far have reported only one or two integrated tasks, new platforms offering multiple integrated functions and rapid analysis of highly complex biological samples can be expected in the near future.

In this work, microchips made of epoxy photoresist SU-8 with a monolithically integrated ESI/MS interface were used for the electrokinetic (MCE, cIEF) separation of biomolecules and drug molecules before online ESI/MS detection. The SU-8 microchips are ready for use directly after fabrication with no need for further surface treatment. The pH dependent surface charge of SU-8 allows for both zero net charge in the separation channel during cIEF focusing (between an acidic anolyte and a basic catolyte) and inherent (cathodic) EOF when using high pH buffers in MCE separations or cIEF mobilizations. Furthermore, the possibility to implement monolithically an on-chip ESI emitter at the microchannel outlet allows for rapid and low-cost mass production of identical chips and reproducible performance from chip to chip.

The microchip methods developed in this study were exploited to both proteomics and metabolomics research. For both of these, short analysis times are key. Here, the SU-8 microchip provided MCE separations of tryptic peptides or drug metabolites in less than 10 min or corresponding cIEF separations, including loading, focusing, and mobilization, in 1 to 10 min. Separation was followed by MS or MS/MS detection.

A challenge often encountered in bioanalysis is the limited amount of sample available, but analytical microsystems provide advantages here too. In a typical T-cross microchip, the sample volume injected is determined by the volume of the intersection of the separation and injection channels, and here it was just 75 pL. A problem in MCE that remains to be tackled is that, even though the amount of sample actually analyzed is very small, the amount that must be pipetted to the sample inlet is many times greater (here, approximately 3 μL). Furthermore, although the small injection volumes in MCE provide advantages in terms of low sample consumption, relatively high sample concentrations will usually be needed to meet the requirements of the detector. While relatively good LOD (S/N=3) and LOQ (S/N=10) values (9.3 nM and 31.2 nM for LOD and LOQ, respectively) for 1-hydroxy bufuralol

were achieved in this study, very small injection volumes will often force the use of micromolar concentrations if a preconcentration step is not included. Both on-chip LPME (for pretreatment and preconcentration of metabolites from human urine samples) and on-chip SPE (for preconcentration of small drug molecules) were thus integrated on the SU-8 microchips prior to MCE-ESI/MS.

The small sample volumes required in LPME together with the direct integration on-chip offer advantages in terms of speed of analysis and the facility to perform sample concentration before the analysis simply by adjusting the extraction volumes. On the other hand, SPE is the more commonly used method of sample pretreatment. Here, a methacrylate-based porous polymer monolith was fabricated via laser-induced photopolymerization *in situ* at the injection cross of the separation microchip. The use of photopolymerized monoliths in combination with MS detection is superior to packing with beads or particles because of the simplicity of fabrication and elimination of the risk of the beads or particles leaking toward the MS instrument. It is also possible to tune the porosity of the monolith (by optimizing the exposure time and porogen content) and so reduce the back pressure, while maintaining efficient retention of analytes. Thus, the microchip can be fully operated by electrokinetic flow, a feature that also favors precise flow control and easy valving with negligible dead volume (time lag) as complex interfacing to external pumps is not required. In addition, the surface chemistry of the monolith can be modified to allow selectivity simply by changing the monomer composition.

All in all, this study offers further proof that microchip systems have great potential in modern (bio)analytical chemistry. The future success of these systems in routine analysis would nevertheless require a clear demonstration of the superiority of the technology and robustness as well as sufficiently low cost in daily operation. However, the potential of microanalysis systems might also lay in the possibility of performing fast on-chip extractions followed by very fast, rough separations before or in addition to standard techniques. For this purpose, it is not even necessary to achieve as accurate and robust results as with e.g., standard HPLC-MS systems.

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Research Article

Feasibility of SU-8-based capillary electrophoresis-electrospray ionization mass spectrometry microfluidic chips for the analysis of human cell lysates

Monolithically integrated, polymer (SU-8) microchips comprising an electrophoretic separation unit, a sheath flow interface and an ESI emitter were developed to improve the speed and throughput of proteomics analyses. Validation of the microchip method was performed based on peptide mass fingerprinting and single peptide sequencing of selected protein standards. Rapid, yet reliable identification of four biologically important proteins (cytochrome C, β-lactoglobulin, ovalbumin and BSA) confirmed the applicability of the SU-8 microchips to ambitious proteomic applications and allowed their use in the analysis of human muscle cell lysates. The characteristic tryptic peptides were easily separated with plate numbers approaching 10^6, and with peak widths at half height as low as 0.6 s. The on-chip sheath flow interface was also exploited to the introduction of an internal mass calibrant along with the sheath liquid which enabled accurate mass measurements by high-resolution Q-TOF MS. Additionally, peptide structural characterization and protein identification based on MS/MS fragmentation data of a single tryptic peptide was obtained using an ion trap instrument. Protein sequence coverages exceeding 50% were routinely obtained without any pretreatment of the proteolytic samples and a typical total analysis time from sampling to detection was well below ten minutes. In conclusion, monolithically integrated, dead-volume-free, SU-8 microchips proved to be a promising platform for fast and reliable analysis of complex proteomic samples. Good analytical performance of the microchips was shown by performing both peptide mass fingerprinting of complex cell lysates and protein identification based on single peptide sequencing.

Keywords:
Epoxy photoresist SU-8 / Mass spectrometry / Microfluidics / Proteomics
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1 Introduction

Microfluidic devices have recently emerged as a powerful analytical platform to execute liquid-phase analyses [1]. The miniature format and the capability of handling minute sample amounts result in rapid analyses and significantly reduced costs without compromising the analytical performance [2, 3]. The surface chemistry of microfluidic channels can also be altered in a similar manner to that of conventional silica capillaries in order to implement various electrophoretic separation techniques on chips [4, 5] or to anchor solid phase supports for on-chip chromatographic separations [6, 7]. Especially polymer microdevices have recently become a convenient bioanalytical platform as they provide all these advantages and can be regarded as disposable, low-cost devices.

Thanks to its high sensitivity and selectivity, ESI/MS is presently one of the main techniques in bioanalysis and lays the foundation for proteomics research [8]. ESI/MS also greatly benefits from miniaturization and interfacing microfluidic devices with ESI/MS is thus of growing interest [9]. Downscaling ESI to a nanospray regime allows a reduction in the applied flow rates and consequently, an

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Abbreviations: EIE, extracted ion electropherogram; **ES,** electrospray; **SLI,** sheath liquid inlet

increase in detection sensitivity compared to conventional ESI. In addition, parallel production of sharp, accurately defined emitters by microfabrication means ensures improved reproducibility compared to that of individually prepared nanospray needles. Much effort has gone into implementation of miniaturized ESI emitters on silicon, glass and polymers and these are comprehensively covered by many recent reviews [1, 10–13]. However, realization of on-chip separation before ESI/MS detection requires sophisticated microfabrication techniques to enable dead-volume-free interfacing of the separation column with the emitter. Apart from some recent designs [14–19], most on-chip electrophoretic or liquid chromatographic separation devices have been combined with MS through external nanospray capillaries manually inserted at the end of the separation channel [20–24]. The more reproducible approach, however, is to pattern the emitter directly at the end of the separation column by microfabrication means [14–19] and thereby avoid the dead volumes in fluidic junctions, which may drastically reduce the separation efficiency. Here, polymer microfabrication technology offers great opportunities. In comparison to silicon and glass processing, patterning of polymer substrates is relatively rapid and inexpensive and sharp ESI emitter structures can be easily patterned as integral parts of separation microdevices [25, 26]. Lithographically defined polymer materials (*e.g.* negative photoresist SU-8), in particular, enable mass production of very complex yet accurately defined microstructures [26]. As a full-wafer process, photolithography also enables fabrication of highly integrated microdevices during a single photoexposure step [26].

The potential of microfluidic chips in proteomics research has been shown, for instance, by coupling LC microchips to MS [14, 15, 27]. These LC-ESI/MS microchips have been capable of separating tryptic peptides of BSA at low femtomole detection levels, but the speed of analysis has remained similar to that of conventional analysis systems, *i.e.* in the range of tens of minutes to one hour *per* sample. This clearly compares unfavorably to the expected rapidity of microchip analysis and does not provide significant improvement with respect to the sample throughput as compared to conventional instrumentation. Integrated CZE-ESI/MS microchips, in turn, have been shown to provide better performance in terms of sample throughput. Mellors *et al.* [18] recently reported a fully integrated CZE-ESI/MS microchip made of glass and capable of analyzing complex protein digests with a fairly high throughput (approximately 3 min *per* sample on a 20.5-cm-long separation channel). Later the same microchip was also utilized for single cell lysis followed by ESI/MS detection of hemoglobin originating from the lysed cells [28]. In another work, direct ESI/MS analysis of a BSA tryptic digest, without prior separation, was demonstrated using an integrated glass CZE-ESI/MS microchip [17]. However, chromatographic or electrophoretic separation prior to MS detection adds in specificity of the analysis because of the possibility to use the migration time data as an additional identification parameter, along with m/z for individual analytes. In addition, fabrication of glass microchips often involves certain manual and serial processing steps, which limit their mass production and batch-to-batch reproducibility. For example, cutting of the emitter by a dicing saw [18, 28] or manual pulling of the emitter to a tip shape [17] was required to implement ESI emitters on the above-mentioned separation microchips.

In this work, integrated CZE-ESI/MS microchips were fabricated from SU-8 polymer so that all critical structures were simultaneously patterned by photolithography [29, 30]. SU-8 microfabrication technology relies on high-accuracy full-wafer photolithography, an approach that enables parallel production of tens of chips with identical features at the same time and thus very good reproducibility from batch-to-batch, similar to SU-8-based direct spray ESI emitters [31]. The applicability of the microchips to protein identification based on peptide mass fingerprinting and peptide sequencing was demonstrated by combining microchip CZE separation with online ESI/MS or MS/MS detection of tryptic peptides. Eventually, the SU-8 CZE-ESI/MS microchip was utilized in the analysis of human muscle cell lysates.

2 Materials and methods

2.1 Chemicals

BSA, cytochrome C from bovine heart, ovalbumin from hen egg white, and β-lactoglobulin from bovine milk, as well as angiotensin I, angiotensin II and bradykinin fragment 1–5 were all purchased from Sigma-Aldrich (Steinheim, Germany). Sequencing grade modified porcine trypsin (Promega, Madison, WI, USA) was used as the digestion enzyme. Methanol and acetic acid were from Mallinckrodt Baker B.V. (Deventer, The Netherlands). Trizma base, ammonium acetate, ammonium formate and formic acid were from Sigma-Aldrich and ACN was from Rathburn Chemicals (Walkerburn, Scotland). Water was purified with a Milli-Q water purification system (Millipore, Molsheim, France). All reagents and solvents were of analytical or HPLC grade.

Negative photoresist SU-8 50 (Microchem, Newton, MA, USA) was purchased from Microresist Technologies GmbH (Berlin, Germany) and PDMS (Sylgard 184, Dow Corning, Midland, MI, USA) from VWR International Oy (Espoo, Finland).

2.2 Standard protein digestion

Incubation of the proteins with trypsin was performed according to a standard protocol. Briefly, 4 μL of enzyme solution (0.35 mg/mL in 50 mM acetic acid) was added to 4 μL of protein solution (10 mg/mL in water). The mixture

was kept at room temperature for 10 min followed by addition of 15 μL of 0.1 M Tris (pH 9.2) with 10% ACN to give a final protein concentration of 1.7 mg/mL and an enzyme:protein ratio of 1:29. Incubation was performed at 37°C for 18 h. Blank control samples were prepared by replacing the protein or the enzyme solution with water or 50 mM acetic acid, respectively. The control blank samples were then incubated in the same manner as described previously. All samples were analyzed without further modifications or prior pretreatment.

2.3 Preparation of human muscle cell lysates

Human muscle cells were lysed according to standard protocol in RIPA buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 tablet protease inhibitor cocktail). The lysed cells were stored in a freezer (−20°C) until used. Before digestion the cell lysate was vortexed for 2 min followed by centrifugation at 13 200 rpm for 10 min. The supernatant, containing only soluble proteins, was digested with trypsin for 18 h at 37°C. The incubation mixture contained 25.8% cell lysate v/v and 0.09 μg/μL trypsin in 0.1 M Tris buffer (pH 9.2) with 10% ACN. No further modifications or pretreatments were performed before analysis.

2.4 Microchips designs

The microchips (Fig. 1) comprising a monolithically integrated injection and separation unit, a sheath flow interface and an ESI emitter were fabricated entirely from epoxy photoresist SU-8 using photolithography and adhesive bonding techniques as reported earlier. [16, 30] The effective separation lengths of the CZE-ESI/MS chips were 4 or 2 cm. Other microchannel dimensions are given in detail in Fig. 1. Before use, PDMS sheets with 2 mm inlet holes were attached on top of the SU-8 chip to increase the inlet volumes.

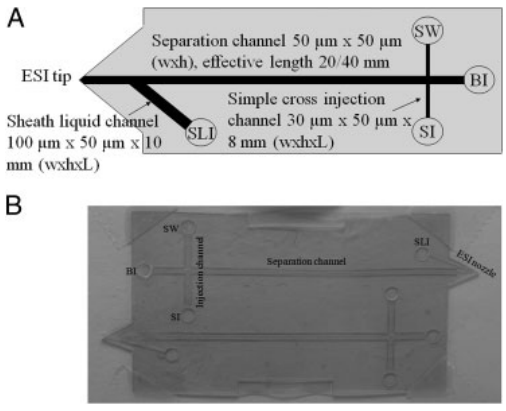

Figure 1. Schematic view of the fluidic design (A) and photograph (B) of the SU-8 CZE-ESI/MS microchip (dimensions not to scale). BI = buffer inlet, SI = sample inlet, SW = sample waste, SLI = sheath liquid inlet.

2.5 Microchip CZE

The samples were injected electrokinetically (25 s) in pinched injection mode. An electric field of 1000–1250 V/cm was applied between the sample inlet and the sample waste, while a small focusing potential was applied to the buffer inlet (Fig. 1). During injection, the sheath liquid inlet (SLI) was floating so that no spray was produced. The CZE separations were performed in cathodic mode using electric field strengths of 375–650 V/cm between the buffer inlet and SLI. The BGE was 30 mM ammonium acetate (pH 7.1) or ammonium formate (pH 6.3) with 50% methanol, while the sheath liquid was a methanol–water (80:20) solution with 1% acetic or formic acid, respectively.

2.6 MS

The SU-8 microchips were coupled to two different MS instruments. An Agilent 6330 MS ion trap MS (Agilent Technologies, Santa Clara, CA, USA) equipped with an *xyz*-aligning stage and a CCD camera was used for rapid analysis of the protein digests by MS and MS/MS, and a Q-TOF Micro MS (Waters/Micromass, Manchester, UK) was used for high-resolution MS measurements. In both cases, an external power supply (Micralyne, Edmonton, Canada) was used for application of the separation and electrospray (ES) voltages through platinum wires placed in the microchannel inlets. The ES voltage also served as the counter voltage for the CZE separation.

Typically, an ES voltage of 3.6 kV (relative to MS) was applied to the SLI. The separation current (typically 20–40 μA) was divided at the sheath flow intersection so that the ES current was 50–300 nA and the excess current was led to ground through a 50 MΩ (ion trap) or 100 MΩ (Q-TOF) resistor coupled in parallel with the ES voltage power supply.

The ion trap MS was operated in positive ion mode with a capillary voltage of −1.6 kV, end-plate offset of −500 V, and a trap drive value of 94.6. Nitrogen (Nitrogen generator system, Parker, Cleveland, OH, USA) was used as the drying gas (4.0 L/min, +70°C). In MS mode, the data was acquired over a mass range of *m/z* 100–2200 with a maximum accumulation time of 100 ms. In MS/MS mode, collision-induced dissociation was performed for the selected precursor ions using a fragmentation amplitude of 2.00 V and an isolation width of 2.0 Da. Data analysis software was used for acquisition and data processing.

The Q-TOF micro instrument was operated in positive ion mode with sample cone voltage of 50.0 V and extraction cone voltage of 2.0 V. The TOF parameters were: 5630 V flight tube, 842 V pusher, 640 V puller. Nitrogen produced by a high-purity nitrogen generator (Peak Scientific, Renfrewshire, Scotland) was used as the cone gas (40 L/h). The MS data was acquired over a mass range of m/z 300–1500 in continuum mode with a scan time of 0.2 s and an inter-scan delay of 0.1 s. For high-resolution MS, bradykinin fragment 1–5 (m/z 573.3149) dissolved in the sheath liquid (5 μM) was used as an internal mass calibrant. The MassLynx 4.0 software was used for data acquisition and processing.

The theoretical molecular masses (M_r), pI values and amino acid sequences of the tryptic peptides were derived from the MASCOT® online search engine (www.matrixscience.com) using the Swiss-Prot database therein. Peaks observed at m/z within $\pm$1.0 Da (tryptic peptides originating from standard proteins) or $\pm$1.2 Da (tryptic peptides from the cell lysates) of their theoretical values were considered as positive matches and maximum one missed cleavage was allowed.

2.7 Analysis of human muscle cell lysates

Peptide mass fingerprinting was performed in order to identify the potential proteins from the human muscle cell lysates. First, the tryptic digests of the cell lysates were analyzed by traditional HPLC-ESI/MS (for experimental set-up, see the Supporting Information) to get a list of cellular proteins potentially present in the cell lysate. Next, the same samples were analyzed by microchip-CE-ESI/MS on the ion trap MS (similarly to the tryptic digests of the standard proteins) and the data was searched for the proteins identified by HPLC-ESI/MS. Only proteins that were identified by both techniques were considered as positive matches.

3 Results and discussion

In this work, SU-8 microfabrication technology, including photolithographic patterning of a microchannel network (Fig. 1) as well as enclosure of the microstructures by SU-8 adhesive bonding [29], was utilized for fabrication of microchip analysis systems for proteomics research. A microfluidic platform was developed comprising a sample introduction unit and an electrophoretic separation channel with a monolithically integrated ESI emitter as well as an auxiliary channel for the introduction of sheath liquid and internal mass calibrant (Fig. 1). The main advantage of the full-wafer SU-8 microfabrication process is that both separation and injection microchannels as well as an ESI emitter tip can be fabricated at the same time during a single photoexposure step. The chip-to-chip reproducibility is therefore high and manufacturing costs low, since a number of microchips can be implemented on the same wafer [16]. Aligning of the different SU-8 layers also exploits lithographic accuracy and allows sharp and precisely defined emitter structures to be patterned in the end of the separation microchannels without manual postprocessing. Here, the applicability of the SU-8 microchips to proteomics research was demonstrated by performing protein identification based on rapid CZE separation of tryptic peptides prior to their online MS identification and MS/MS characterization. The SU-8 microchips were coupled to two different MS instruments, namely an ion trap and a Q-TOF MS, both of which record a broad mass range at high speed and thus provide enough data points for reliable recording of mass spectra from narrow electrophoretic peaks. The microchip method was validated with the help of selected standard proteins (cytochrome C, β-lactoglobulin, ovalbumin and BSA) that sufficiently differed in size, pI value and hydrophilicity (Table 1) and represented proteins of different physical and chemical properties. Eventually, separation of tryptic peptides followed by peptide mass fingerprinting and identification of proteins from human muscle cell lysate samples was demonstrated using the SU-8 CZE-ESI/MS microchip.

3.1 Protein identification by peptide mass fingerprinting

Before analysis, the CZE separation voltages and the MS parameters were optimized with the help of selected peptide standards, *i.e.* angiotensin I and II. The migration times of angiotensin I and II were 72.0 and 81.1 s, respectively. The run-to-run ($n = 3$) repeatability was determined by utilizing

Table 1. Summary of the microchip CZE-ESI/MS analyses of the tryptic digests of cytochrome C, β-lactoglobulin, ovalbumin and BSA

Protein	M_r (kDa)	pI	Amino acids	GRAVY[a)]	Number of peptides[b)]	Sequence cov. (%)[b)]	Probability score[b)]	Expect. value[b)]
Cytochrome C (CYC_BOVIN)	12	9.52	104	−0.866	10	67	87	2.0×10^{-4}
ß-Lactoglobulin (LACB_BOVIN)	18	4.76	162	−0.167	17	68	97	1.8×10^{-5}
Ovalbumin (OVAL_CHICK)	43	5.20	386	−0.006	19	59	93	5.4×10^{-5}
BSA (ALBU_BOVIN)	69	5.78	583	−0.476	46	54	177	2.0×10^{-13}

a) Grand average of hydropathicity index.
b) Based on Swiss-Prot database search by MASCOT online search engine.

angiotensin II as an internal standard for angiotensin I. The measured repeatabilities for the migration time and the peak area were 4.7 and 3.2%, respectively. The BGE composition was then optimized separately for each proteolytic sample, with respect to the electrolyte type (ammonium acetate or ammonium formate) and concentration as well as the amount of methanol in the BGE. For most protein digests, a 30 mM ammonium acetate solution containing 50% methanol was shown to provide the best resolving power, yet ammonium formate solution of the same strength allowed the best resolution of ovalbumin tryptic peptides. Typically, a number of singly or doubly charged tryptic peptides were identified and sequence coverages between 50 and 70% were routinely obtained for all selected proteins (Table 1). A representative separation of the tryptic peptides observed for cytochrome C with their extracted ion electropherograms (EIE) is given in Fig. 2. Tables S1–S3 in the Supporting Information give detailed lists of all peptides observed for ovalbumin, ß-lactoglobulin and BSA digests, respectively.

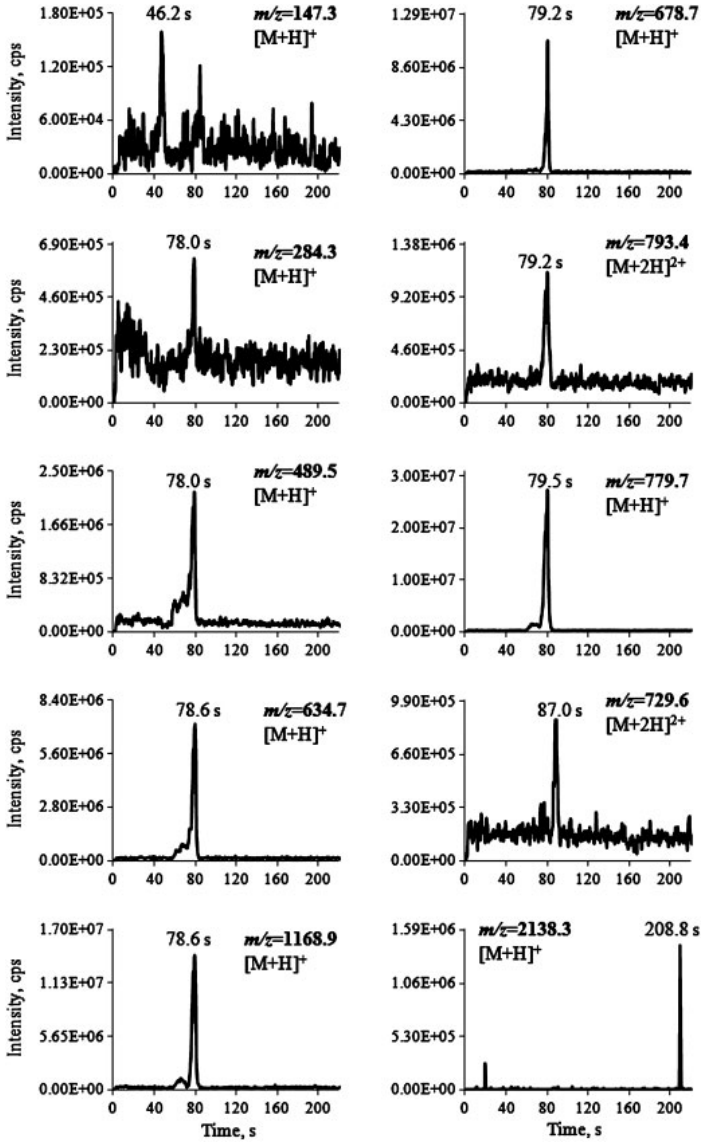

Figure 2. EIEs from the microchip CZE-ESI/MS analysis of the cytochrome C tryptic digest on the ion trap MS. The separation BGE was 30 mM ammonium acetate with 50% methanol and the sheath liquid was 80% methanol with 1% acetic acid. Electric field strengths during injection and separation were 1000 and 375 V/cm, respectively, and the effective separation length was 4 cm.

Thanks to the straightforward integration of all unit operations (*i.e.* sample introduction, separation and ESI) on the same microchip, the fluidic junctions were dead volume free, which resulted in negligible peak broadening and a high number of theoretical separation plates (*N*). At the best, peak widths at half height (w_h) down to 0.6 s were reached for the tryptic peptides, which gave theoretical plate numbers (*N*) as high as 6.7×10^5. Most importantly, such efficient separation together with reliable identification of the protein in question was achieved within very short analysis times (2.5–6.3 min on a 4-cm-long separation channel) and with minimal manual workload. Thanks to the very small injection volume of only 45 pL (as *per* volume of the intersection of the injection and separation channels), the digestion matrix containing relatively high electrolyte concentrations could be injected as such, without any need for sample pretreatment before analysis. The amount of protein injected *per* analysis was as low as 1.0–6.3 fmol, as *per* initial protein concentration (20–140 μM) in the digestion matrix, showing that the tryptic peptides could be detected with reasonably high sensitivity regarding proteomics applications.

Although surface contamination through nonspecific adsorption of biomolecules is often reported for polymer microchips, here adsorption of the tryptic peptides to SU-8 was considered negligible since blank runs did not show any memory effects. The addition of small amounts of organic solvents, as was done here, or surfactants to the BGE has also been shown to significantly reduce nonspecific interactions between the SU-8 surface and the analytes of interest. [16]

The layout of the SU-8 microchip (Fig. 1) is also well suited for introduction of an internal mass calibrant together with the sheath liquid in order to allow exact mass measurements on, for instance, Q-TOF instruments. Here, bradykinin fragment 1–5 (*m/z* 573.3149) was chosen as an

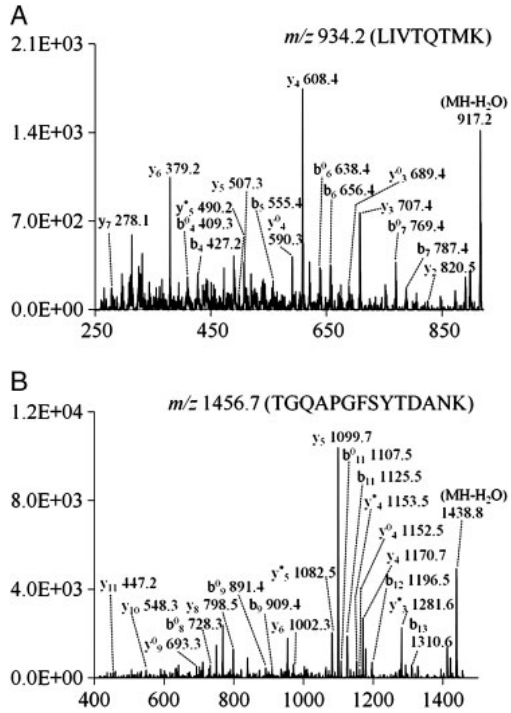

Figure 4. ESI-collision-induced dissociation spectra of selected singly charged tryptic peptides of (A) β-lactoglobulin (*m/z* 934.2) and (B) cytochrome C (*m/z* 1456.7) as precursor ions from the microchip CZE-ESI/MS/MS on the ion trap MS. The separation BGE was 30 mM ammonium acetate with 50% methanol and the sheath liquid was 80% methanol with 1% acetic acid. Electric field strengths during injection and separation were 1000 and 375 V/cm, respectively, and the effective separation length was 4 cm. The fragmentation amplitude was 2.0 V.

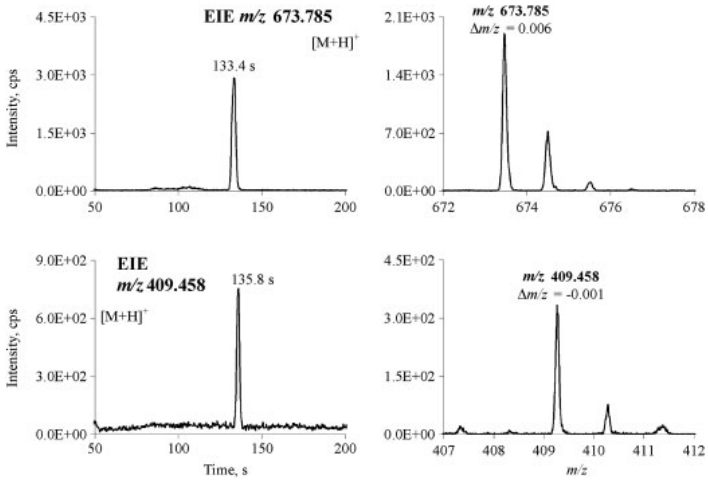

Figure 3. Selected EIEs and corresponding high-resolution mass spectra from the microchip CZE-ESI/MS analysis of β-lactoglobulin on the Q-TOF MS. The separation BGE was 30 mM ammonium acetate with 50% methanol and the sheath liquid was 80% methanol with 1% acetic acid and 5 μM bradykinin fragment 1–5 as sheath liquid. Electric field strengths during injection and separation were 1250 and 400 V/cm, respectively, and the effective separation length was 4 cm.

internal mass calibrant and was applied along with the sheath liquid at a concentration of 5 μM. In this manner, it did not interfere with the analytes of interest during separation, but enabled identical online mass calibration between all separated analytes. Figure 3 shows the EIEs and mass spectra of selected peptides from the β-lactoglobulin tryptic digest and Table S4 in the Supporting Information lists the peptides observed from the cytochrome C tryptic digest analyzed by microchip CZE-ESI coupled to a high-resolution Q-TOF MS. Both the EIEs and mass spectra show good S/N and clearly identify the peptide fragments with a typical mass error around 10 ppm (Fig. 3 and Table S4).

3.2 Protein identification by MS/MS

An obvious advantage of the rapid analysis facilitated by the microchip CZE-ESI/MS is the ability to perform MS/MS on very small quantities of the protein digest. In addition to accurate mass determination by high-resolution MS instruments, MS/MS provides detailed structural information on the peptides to facilitate protein identification based on peptide sequencing. In this work, MS/MS characterization of tryptic peptides, first separated by on-chip CZE, was done on the ion trap instrument. The MS/MS spectra of selected tryptic peptides from β-lactoglobulin and cytochrome C digests are given in Fig. 4A and B, respectively. The product ion spectra of the selected precursor peptides are characterized by the easily identifiable y and b series fragment ions. Both cytochrome C and ß-lactoglobulin could be accurately identified by the MASCOT® [32] online search engine based on MS/MS fragmentation data of only a single tryptic peptide of each, *i.e.* m/z 1456.7 for cytochrome C and m/z 934.2 for ß-lactoglobulin. The MASCOT scores, indicating identity or extensive homology to the proteins in question, were 67 (expect. value 8.3×10^{-5}) and 42 (expect. value 0.028) for cytochrome C and ß-lactoglobulin, respectively.

3.3 Analysis of human muscle cell lysates

The applicability of the SU-8 CZE-ESI/MS microchip to the analysis of authentic biological samples was shown by analyzing a tryptic digest of human muscle cell lysates. Regardless of the complexity of the human cellular matrix seven proteins of different size, p*I* value and hydrophilicity were identified from the cell lysates (Table 2). The MASCOT® probability-based scores [32] ranged from 102 to 211 indicating significant homology between detected and theoretical tryptic peptides and sequence coverages as good as 93% were received. The EIEs of the tryptic peptides of an actin related protein Q96HG5, highly expressed in muscle cells, are shown in Fig. 5 while Tables S5–S10 in the Supporting Information give detailed lists of the tryptic peptides observed for all other identified proteins from the human muscle cell lysates. All proteins listed in Table 2 were also identified by in-house HPLC-MS analysis of the same sample. A conventional HPLC-MS analysis was carried out with a view to confirm the positive matches because data-dependent MS/MS analysis of the cell lysate was not feasible on microchips. Namely, the acquisition rate of the current MS instrumentation in MS/MS mode is not high enough for reliable sampling of the very narrow electrophoretic peaks (<1 s) characteristic of microchip CE. Therefore, the time constant of the MS detector often becomes a limiting factor in microchip CE-ESI/MS analysis [18, 33] with respect to thorough MS/MS characterization in particular, unless nonscanning instruments with high acquisition rate (*e.g.* TOF-MS) are used. In addition to comparison with HPLC-MS, rigorous screening against blank samples was carried out in order to eliminate potential false positives. When compared to the HPLC-MS method the microchip method is very fast and requires a much smaller amount of sample (<3 μL) for analysis. In microchip CZE-ESI/MS analysis all peptides migrated within 98 s on the 2-cm-long separation microchannel (Table 2), while the HPLC-MS method requires 20 μL of sample and takes tens of minutes *per* one run.

Table 2. Proteins identified from the digested human muscle cell lysate by microchip CZE-ESI/MS analysis on the ion trap MS

Protein		M_r (kDa)	*pI*	Amino acids	GRAVY[a)]	Number of peptides[b)]	Sequence coverage (%)
Q70VV1	Rhesus blood group D antigen	5.6	6.51	48	0.085	5	85
Q6PCD6	GLS2 protein	6.6	11.35	61	−1.075	8	93
Q8N9L7	CDNA FLJ36925 fis, clone BRACE2005169	13.5	9.8	120	−0.756	8	70
CAF00094	DNAJ (Hsp40) homolog, subfamily C member 11	14.4	5.22	119	−1.071	10	65
Q96HG5	Actin beta, (fragment)	41.0	5.56	368	−0.202	17	48
JC4775	Interferon-induced double-stranded RNA activated protein	57.5	5.83	504	−0.679	23	32
Q6PIC7	MAP7 domain-containing protein	74.1	8.68	660	−1.115	21	19

The separation BGE was 30 mM ammonium acetate with 50% methanol and the sheath liquid was 80% methanol with 1% acetic acid. Electric field strengths during injection and separation were 1000 and 650 V/cm, respectively, and the effective separation length was 2 cm.

a) Grand average of hydropathicity index.

b) Based on Swiss-Prot database search by MASCOT online search engine.

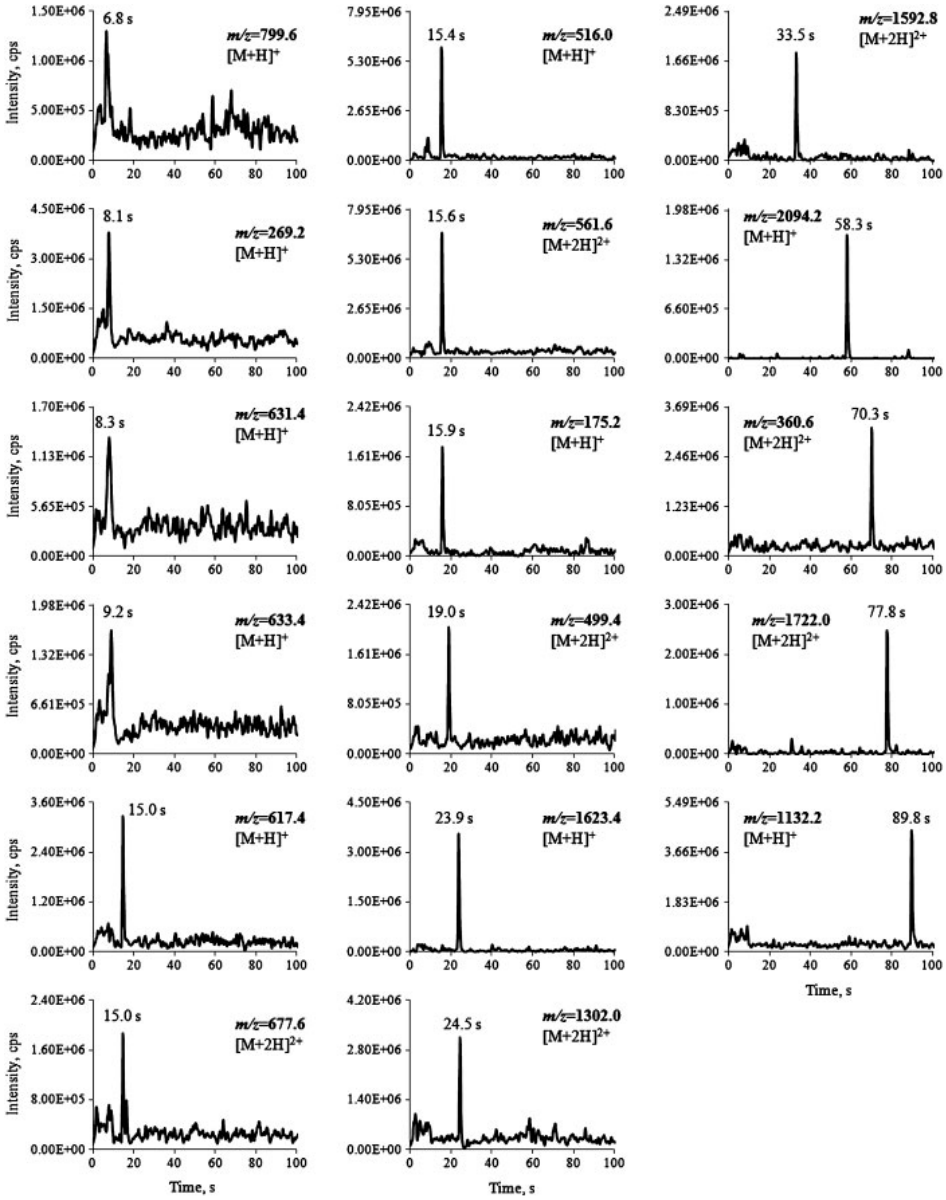

Figure 5. EIEs of the tryptic peptides from the protein Q96HG5 of the human muscle cell lysate analyzed by microchip CZE-ESI/MS on the ion trap MS. The separation BGE was 30 mM ammonium acetate with 50% methanol and the sheath liquid was 80% methanol with 1% acetic acid. Electric field strengths during injection and separation were 1000 and 650 V/cm, respectively, and the effective separation length was 2 cm.

4 Concluding remarks

The microchip methods developed in this study were shown to be efficient tools for proteomics research. The SU-8 microchip CZE-ESI/MS analysis including rapid (<10 min) CZE separation of tryptic peptides and their high-resolution MS or MS/MS identification, was first validated with the help of selected, biologically important proteins. Regardless of the protein properties (size, hydrophilicity, p*I* value), the characteristic peptide fragments were easily separated with

reasonably high sensitivity (at the femtomole level) and with sequence coverages better than 50% and plate numbers approaching 10^6. Next, the microchips were applied to the analysis of authentic biological samples by analyzing tryptic peptides originating from human cell lysates. Fast and efficient separation of a number of tryptic peptides facilitated reliable identification of several human proteins from the complex cellular matrix by microchip CZE-ESI/MS. The microchips also showed good repeatability as indicated by low RSD for both the migration time (RSD = 4.7%) and peak area (RSD = 3.2%). Moreover, the SU-8 microfabrication technology exploited in the chip fabrication not only enables production of accurately defined microstructures but also low-cost mass production of a number of chips with identical features and thus, highly reproducible performance from chip-to-chip. This is the main advantage of the fully integrated SU-8 CZE-ESI/MS microchips over other thus far published CZE-ESI/MS microchips. Inevitably, the speed of microchip CZE-ESI/MS analysis is another indisputable reward as against both conventional and microchip LC-ESI/MS systems.

The Academy of Finland (121882) and the graduate school in electrical and communication engineering provided financial support. Dr. Marc Baumann and Mr. Rabah Soliymani from the Protein Chemistry Core Facility, University of Helsinki kindly provided the cell lysate samples and together with Professor Seppo Auriola from the Department of Pharmacy, University of Eastern Finland gave useful advice regarding protein database searches.

The authors have declared no conflict of interest.

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Supporting information

Analysis of human cell lysates by Rapid Capillary Electrophoresis-Electrospray Ionization Mass Spectrometry on SU-8 Microfluidic Chips

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1. HPLC-MS experiments

An Agilent HP 1100 HPLC system equipped with an Atlantis^{TM} dC_{18} column (2.1 x 50 mm, 3 µm; Waters) was used for the separation of tryptic peptides from a digested human muscle cell lysate sample. The injection volume was 20 µL and the eluent flow rate was 450 µL/min. The aqueous phase (A) contained 5 mM ammonium acetate and the organic phase (B) was methanol. The gradient profile was from 5 to 95% B in 15 min followed by 95% B for 5 min.

The HPLC system was coupled to an API3000 triple quadrupole (QqQ) mass spectrometer (Perkin Elmer Sciex, Concord, ON, Canada) with a commercial turbo electrospray ionization (ESI) source. The 450 µL/min flow from the column was split 1:10 prior to MS detection. The MS was operated in positive ion mode with an electrospray voltage of 5500 V and scanning a mass range of *m/z* 300-2000 (2.5 s/scan). The main ion optics were: declustering potential (DP) 30 V, focusing potential (FP) 200 V, entrance potential (EP) 10 V, and collision exit potential (CE) 30 V. Nitrogen (Whatman 75-72 nitrogen generator; Haverhill, MA) was used as the curtain and turbo gas while compressed air (Atlas Copco air dryer, Wilrijk, Belgium) was used as the nebulizer gas. The flow rates of the curtain and nebulizer gases were 1.44 and 1.53 L/min respectively and the source temperature was 280°C. Data was processed with Analyst1.4 software.

2. Tables

Table S-1. The peptides observed in the microchip CZE-ESI/MS analysis of the tryptic digest of ovalbumin listed as per their migration time together with mass variation between the observed and calculated *m/z*, theoretical molecular mass and position in the amino acid sequence. The BGE was 30 mM ammonium formate with 50% methanol and the sheath liquid was 80% methanol with 1% formic acid. Electric field strengths during injection and separation were 1000 V/cm and 375 V/cm, respectively, on the ion trap MS and the effective length of the separation microchannel was 4 cm.

Migration time (s)	Observed ions		$\Delta m/z$	Theoretical mass (Da)	Position in sequence
	$[M+H]^+$	$[M+2H]^{2+}$			
8.9	1688.2		0.4	1686.8	127-142
14.5	1774.3		0.4	1772.9	323-339
31.9		1382.7	0.0	2763.3	59-84
43.6	535.5		-0.7	535.2	287-290
45.1	1209.1		-0.4	1208.5	190-199
54.3	631.6		-0.7	631.3	182-186
55.1	409.4		0.2	408.2	59-61
62.3		1140.8	-0.3	2280.2	85-104
73.3	277.1		-1.0	277.1	227-228
73.3	388.3		0.0	387.3	277-279
73.4	260.1		-0.1	259.2	278-277
76.0		1737.8	0.0	3473.6	190-218
78.1		1544.2	0.0	3086.5	200-226
107.6	1581.5		-0.2	1580.7	264-276
126.8		1646.8	-0.5	3292.6	291-322
148.8	2025.7		-1.0	2026.1	111-126
154.6		1537.4	0.1	3072.6	159-186
191.2	1730.9		-1.0	1730.9	370-385
306.5	1344.9		-0.8	1344.7	370-381

Table S-2. The peptides observed in the microchip CZE-ESI/MS analysis of the tryptic digest of β-lactoglobulin listed as per their migration time together with mass variation between the observed and calculated *m/z*, theoretical molecular mass and position in the amino acid sequence. The BGE was 30 mM ammonium formate with 50% methanol and the sheath liquid was 80% methanol with 1% acetic acid. Other injection and separation conditions as per Table S1.

Migration time (s)	**Observed ions**			**Δ*m/z***	**Theoretical mass (Da)**	**Position in sequence**
	$[M+H]^+$	**$[M+2H]^{2+}$**	**$[M+3H]^{3+}$**			
4.2	247.7			-0.6	247.3	76-77
25.2	1964.9			0.7	1963.2	84-100
64.2	331.4			0.0	330.4	139-141
64.8	837.9			-0.1	837.0	142-148
66.6	674.9			0.1	673.8	78-83
68.4		561.7		-0.4	1122.2	61-69
69.0	933.9			-0.3	933.2	1-8
82.2	673.8			0.0	672.8	9-14
84.6	573.7			0.0	572.7	71-75
88.8	409.4			0.0	408.4	136-138
90.0			826.7	-0.3	2477.9	142-162
95.4			892.1	-0.9	2676.1	102-124
97.8	1065.8			-0.4	1065.2	92-100
99.6	916.2			-0.8	916.0	84-91
103.8	904.9			0.8	903.1	76-83
109.2		1157.8		0.0	2313.6	41-60
148.8	1245.8			-0.5	1245.3	125-135

Table S-3. The peptides observed in the microchip CZE-ESI/MS analysis of the tryptic digest of BSA listed as per their migration time together with mass variation between the observed and calculated *m/z*, theoretical molecular mass and position in the amino acid sequence. The BGE was 30 mM ammonium formate with 50% methanol and the sheath liquid was 80% methanol with 1% acetic acid. Other injection and separation conditions as per Table S1.

Migration time (s)	Observed ions	Δ*m/z*	Theoretical mass (Da)	Position in sequence	Migration time (s)	Observed ions	Δ*m/z*	Theoretical mass (Da)	Position in sequence
49.2	509.6 (1+)	0.3	508.3	558-561	101.2	500.5 (1+)	0.3	499.2	25-28
52.5	589.6 (2+)	0.3	1176.6	300-309	101.7	517.6 (1+)	0.3	516.3	281-285
59.3	723.7 (2+)	0.3	1444.8	157-167	102.5	789.9 (1+)	0.4	788.5	257-263
88.3	733.8 (2+)	0.4	1464.7	456-468	102.9	649.7 (1+)	0.4	648.3	223-228
88.5	475.6 (1+)	0.3	474.3	242-245	102.9	508.5 (1+)	0.3	507.2	229-232
88.6	689.8 (1+)	0.4	688.4	236-241	103.2	1002.9 (1+)	0.3	1001.6	598-607
88.7	545.7 (1+)	0.4	544.3	101-105	103.2	1283.9 (1+)	0.2	1282.7	361-371
89.0	432.5 (1+)	0.3	431.2	456-459	103.4	712.8 (1+)	0.4	711.4	29-34
89.1	572.7 (1+)	0.3	571.4	219-222	103.5	946.2 (2+)	0.3	1889.8	101-117
89.4	1640.1 (1+)	0.2	1638.9	437-451	105.5	660.7 (1+)	0.4	659.3	490-495
90.3	388.5 (1+)	0.3	387.2	545-547	105.7	1014.9 (1+)	0.3	1013.6	549-557
90.5	383.4 (1+)	0.2	382.2	264-266	112.0	752.9 (1+)	0.5	751.4	341-346
90.5	331.4 (1+)	0.2	330.2	372-374	140.9	1231.9 (2+)	0.2	2461.4	3-23
90.5	821.2 (1+)	0.7	819.5	483-489	162.9	1351.9 (2+)	0.8	2700.2	460-482
90.6	347.4 (1+)	0.2	346.2	496-498	194.6	1889.4 (1+)	0.5	1887.9	169-183
90.7	404.4 (1+)	0.2	403.2	452-455	201.3	1884.9 (1+)	0.0	1883.9	281-297
90.8	439.5 (1+)	0.3	438.2	434-436	229.6	1039.1 (2+)	0.1	2075.9	267-285
91.0	391.4 (1+)	0.2	390.2	246-248	240.1	979.1 (2+)	0.6	1955.0	319-336
91.1	1143.0 (1+)	0.3	1141.7	548-557	241.5	1386.7 (1+)	0.1	1385.6	286-297
91.6	537.6 (1+)	0.3	536.3	157-160	242.6	886.8 (1+)	0.4	885.4	131-138
99.3	1017.9 (2+)	0.3	2033.1	588-607	374.1	318.4 (2+)	0.7	633.4	20-24
99.4	741.2 (3+)	0.5	2219.1	529-547	374.6	362.4 (3+)	0.5	1082.6	161-168
99.4	1480.0 (1+)	0.2	1478.8	421-433	375.5	333.5 (2+)	0.3	664.4	156-160
101.0	1512.0 (1+)	0.2	1510.8	438-451					

Table S-4. The peptides observed in the microchip CZE-ESI/MS analysis of the tryptic digest of cytochrome C listed as per their migration time together with mass variation between the observed and calculated *m/z*, theoretical molecular mass and position in the amino acid sequence. The BGE was 30 mM ammonium acetate with 50% methanol and the sheath liquid was 80% methanol with 1% acetic acid and 5 μM bradykinin fragment 1-5. Electric field strengths during injection and separation were 1000 V/cm and 400 V/cm, respectively, on the Q-TOF MS and the effective length of the separation microchannel was 4 cm.

Migration time (s)	**Observed ions**		**Error (ppm)**	**Theoretical mass (Da)**	**Position in sequence**
	$[M+H]^+$	**$[M+2H]^{2+}$**			
42.6		584.8216	10.8	1167.6149	28-38
43.2	284.1754		10.9	283.2644	26-27
43.2	489.2704		-16.6	488.2707	88-91
43.2	634.3887		-6.5	633.3850	9-13
43.2	678.3816		-1.6	677.3748	74-79
43.2	779.4363		-16.3	778.4411	80-86
44.4	261.1590		10.3	260.1485	23-25 or 54-55
44.4		728.8521	17.6	1455.6630	40-53
48.0	361.1729		-29.6	360.1757	89-91
48.0	964.5020		-34.7	963.5277	92-99
50.4	434.1909		5.1	433.1809	101-104

Table S-5. Tryptic peptides of Q70VV1 observed in the microchip CZE-ESI/MS analysis of the digested human muscle cell lysate listed as per their migration time together with mass variation between the observed and calculated *m/z*, theoretical molecular mass and position in the amino acid sequence. The BGE was 30 mM ammonium acetate with 50% methanol and the sheath liquid was 80% methanol with 1% acetic acid. Electric field strengths during injection and separation were 1000 V/cm and 650 V/cm, respectively, on the ion trap MS and the effective length of the separation microchannel was 2 cm.

Migration time (s)	**Observed ions**		**Δ*m/z***	**Theoretical mass (Da)**	**Position in sequence**
	$[M+H]^+$	**$[M+2H]^{2+}$**			
15.9	175.2		0.1	174.1	11-11
24.3		267.2	0.1	532.3	8-11
44.2		1823.2	0.5	3643.9	12-42
52.0	793.8		0.4	792.4	5-10
90.0		368.6	-1.2	736.4	2-7

Table S-6. Tryptic peptides of Q6PCD6 observed in the microchip CZE-ESI/MS analysis of the digested human muscle cell lysate listed as per their migration time together with mass variation between the observed and calculated *m/z*, theoretical molecular mass and position in the amino acid sequence. The BGE was 30 mM ammonium acetate with 50% methanol and the sheath liquid was 80% methanol with 1% acetic acid. Injection and separation conditions as per Table S5.

Migration time (s)	**Observed ions**		**Δ*m/z***	**Theoretical mass (Da)**	**Position in sequence**
	$[M+H]^+$	**$[M+2H]^{2+}$**			
8.0		1007.0	-0.1	2012.1	29-48
8.1		761.8	0.9	1520.7	14-28
15.9	175.2		0.1	174.1	2-2
21.2	1567.8		-0.9	1567.7	49-61
22.1	459.4		0.1	458.3	6-9
24.3		1364.2	1.2	2725.2	38-61
48.7		326.4	-0.5	651.3	1-5
54.0	855.8		0.3	854.5	29-37

Table S-7. Tryptic peptides of Q8N9L7 observed in the microchip CZE-ESI/MS analysis of the digested human muscle cell lysate listed as per their migration time together with mass variation between the observed and calculated *m/z*, theoretical molecular mass and position in the amino acid sequence. The BGE was 30 mM ammonium acetate with 50% methanol and the sheath liquid was 80% methanol with 1% acetic acid. Injection and separation conditions as per Table S5.

Migration time (s)	**Observed ions**		**Δ*m/z***	**Theoretical mass (Da)**	**Position in sequence**
	$[M+H]^+$	**$[M+2H]^{2+}$**			
8.0		432.4	-0.6	863.4	58-65
8.6		413.4	-0.7	825.5	9-15
11.2	1763.0		0.2	1761.8	16-29
15.9	175.2		0.1	174.1	57-57
15.9		1125.0	-0.1	2248.1	58-77
18.1		1606.8	-0.1	3211.7	66-94
24.5	1302.0		-0.6	1301.6	2-12
72.0		1860.6	-0.7	3719.9	78-113

Table S-8. Tryptic peptides of CAF00094 observed in the microchip CZE-ESI/MS analysis of the digested human muscle cell lysate listed as per their migration time together with mass variation between the observed and calculated *m/z*, theoretical molecular mass and position in the amino acid sequence. The BGE was 30 mM ammonium acetate with 50% methanol and the sheath liquid was 80% methanol with 1% acetic acid. Injection and separation conditions as per Table S5.

Migration time (s)	**Observed ions**		**Δ*m/z***	**Theoretical mass (Da)**	**Position in sequence**
	$[M+H]^+$	**$[M+2H]^{2+}$**			
8.2		688.6	-0.5	1375.7	99-109
14.8	1099.0		0.4	1097.6	76-84
15.9	175.2		0.1	174.1	many
19.6		471.4	-0.7	941.5	76-83
20.1		795.8	0.8	1588.8	84-96 or 85-97
25.2	1353.2		-0.5	1352.7	24-35
46.6		421.4	-0.7	841.5	98-104
53.2		447.4	-0.7	893.5	46-52
56.0		1366.0	-0.3	2730.3	1-23
68.5	635.6		-0.8	635.4	32-36

Table S-9. Tryptic peptides of JC4775 observed in the microchip CZE-ESI/MS analysis of the digested human muscle cell lysate listed as per their migration time together with mass variation between the observed and calculated *m/z*, theoretical molecular mass and position in the amino acid sequence. The BGE was 30 mM ammonium acetate with 50% methanol and the sheath liquid was 80% methanol with 1% acetic acid. Injection and separation conditions as per Table S5.

Migration time (s)	**Observed ions**		**Δ*m/z***	**Theoretical mass (Da)**	**Position in sequence**
	$[M+H]^+$	**$[M+2H]^{2+}$**			
7.4		831.8	-0.2	1661.8	143-156
7.9		723.6	0.5	1444.7	180-191
8	679.6		-0.8	679.4	112-117
8	925.0		0.6	923.4	257-263
8		557.4	0.3	1112.5	281-290
8.1		761.8	-0.1	1521.7	284-296 or 118-130
8.3	631.4		1.0	629.4	408-412
8.7	575.6		0.3	574.3	378-382
9.2		633.4	0.2	1264.6	297-307
14.5	486.4		-1.0	486.4	130-133
14.8	1099.0		0.4	1097.6	394-402
15.4	911.2		-0.3	910.5	251-257
15.6		561.6	-0.3	1121.5	310-318
15.9	175.2		0.1	174.1	many
19.6	1076.0		0.5	1074.5	447-455
19.6	1077.0		-0.7	1076.7	109-117
36.6	715.6		0.2	714.4	201-206
40.9	811.8		0.4	810.4	102-108
48.7		697.6	-0.4	1393.6	118-129
56.9	828.6		0.1	827.5	89-96
64.5	823.8		-0.7	823.5	40-46
72.9	839.8		0.4	838.4	499-504
97.4	489.4		1.1	487.3	403-406

Table S-10. Tryptic peptides of Q6PIC7 observed in the microchip CZE-ESI/MS analysis of the digested human muscle cell lysate listed as per their migration time together with mass variation between the observed and calculated *m/z*, theoretical molecular mass and position in the amino acid sequence. The BGE was 30 mM ammonium acetate with 50% methanol and the sheath liquid was 80% methanol with 1% acetic acid. Injection and separation conditions as per Table S5.

Migration time (s)	Observed ions		Δ*m/z*	Theoretical mass (Da)	Position in sequence
	$[M+H]^+$	$[M+2H]^{2+}$			
6.9	947.8		0.4	946.4	344-350
8.0	515.2		-1.1	515.3	186-189
8.0	596.6		-0.7	596.3	291-295
8.0		596.6	0.6	1190.6	224-234
8.3	631.6		0.2	630.4	151-155
8.7		575.6	0.6	1148.6	291-300
9.1		469.4	0.4	936.4	206-214
9.2	633.4		0.1	632.3	368-372
11.4		252.2	0.1	502.3	151-154
13.2	570.6		-0.7	570.3	296-300
15.0		618.4	1.2	1233.6	296-308
15.9	175.2		0.1	174.1	629-629
17.2	573.4		0.0	572.4	331-335
18.8	1149.0		-0.6	1148.6	291-300
20.1		795.8	-0.3	1589.9	235-248
23.9		657.4	1.1	1311.7	249-260
41.5		317.2	0.1	632.3	368-372
42.0	1797.4		-0.6	1797.0	134-150
46.6	421.4		1.2	419.2	224-227
56.0	1366.0		-0.7	1365.7	156-170
63.4	1317.2		0.6	1315.6	341-350

II

Interfacing Microchip Isoelectric Focusing with On-chip Electrospray Ionization Mass Spectrometry

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Abbreviations: AngI, angiotensin I; AngII, angiotensin II; AngIII, angiotensin III; AN, anolyte; BGE, background electrolyte; BI, buffer inlet; BK1-5, bradykinin fragment 1-5; CA, catholyte; EIE, extracted ion electropherogram; µEP, electrophoretic mobility; His, histidine; HV, high voltage; LE, leading electrolyte; MCE, microchip capillary electrophoresis; SI, sample inlet; SLI, sheath liquid inlet; SP6-11, substance P fragment 6-11; TE, terminating electrolyte; TEMED, N, N, N´, N´-tetramethylethylenediamine; tITP, transient isotachophoresis

Abstract

In this work, we demonstrate the interfacing of microchip capillary isoelectric focusing (cIEF) with online mass spectrometric (MS) detection *via* a fully integrated, on-chip sheath flow electrospray ionization (ESI) emitter. Thanks to the pH-dependent surface charge of the SU-8 polymer used for the chip fabrication, cIEF can be successfully run in native SU-8 microchannels without need for any surface pretreatment prior to analysis. On the other hand, the inherent electroosmotic flow (EOF) taking place in SU-8 microchannels at high pH can be exploited to electrokinetic mobilization of the focused pH gradient toward the MS so that no external pumps are required. In addition to direct coupling of a cIEF separation channel to an ESI emitter, we developed a two-dimensional separation chip for two-step, multiplex cIEF-transient-isotachophoretic (tITP) separation. In this case, the cIEF is performed in the first dimension (effective L= 20 mm) and tITP in the second dimension (L=35 mm) followed by ESI/MS detection. As a result, the migration order is affected by both the *pI* values (cIEF) and the intrinsic electrophoretic mobilities (tITP) of the sample components. The selectivity of the separation system was shown to be different from pure cIEF or pure ITP, which at best allowed for baseline separation of two compounds with nearly identical *pI* values. The repeatabilities of the migration times of the two-step cIEF-tITP separation were as good as 3.1-6.8% RSD (n=3). Thanks to the short separation channel, relatively short focusing times of 60-270 s (depending on the applied focusing potential) were sufficient for establishment of the pH gradient and cIEF separation of the sample components, yielding total analysis times (including loading, focusing, and mobilization) well below 10 min.

Keywords: microchip electrophoresis; isoelectric focusing; isotachophoresis; mass spectrometry; microfluidics

1. Introduction

Microchip capillary (zone) electrophoresis (MCE) in combination with microscope-based fluorescence (FL) detection is the gold standard for microfluidic separations thanks to its high speed of analysis and good feasibility for proteomics research [1, 2]. In addition to free zone electrophoresis, capillary isoelectric focusing (cIEF) is another established technique based on separation of zwitter ionic biomolecules (e.g., peptides and proteins) according to their *pI* and followed by electrokinetic or hydrodynamic mobilization of the focused pH gradient toward the detector [3]. Microchip cIEF has also been implemented for rapid separations of proteins, though usually in combination with linear (optical) imaging of the cIEF gradient through the cover layer, without the mobilization step [4-6]. However, a major limitation of optical detection (UV or FL) in proteomics applications is the fact that the ampholytes (required for establishment of the pH gradient) also strongly absorb UV light below 280 nm. This means that only tryptophan and tyrosine containing peptides and proteins can efficiently be detected. Coupling of cIEF to mass spectrometry (MS) detection is thus of great interest to many proteomics applications, where more specific identification of peptides and proteins is needed. However, it is again the ampholytes that challenge the coupling of cIEF to MS because of the ampholytes' tendency to suppress the ionization efficiency of the target compounds [7, 8]. As a result, the potential of microchip cIEF-MS in proteomics research has often been overlooked.

In this study, we demonstrate efficient coupling of microchip cIEF separation to on-chip electrospray ionization (ESI)/MS detection. Specifically, we exploit the parallel microfabrication technology for implementation of fully integrated multidimensional separation systems with dead-volume-free on-chip ESI emitters and sheath flow interfaces out of SU-8 negative photoresist, similar to earlier work [9-15].

Through SU-8 photolithography and adhesive bonding [10, 16], the construction of complex separation platforms with multiple intersecting microchannels is relatively straightforward. Furthermore, zero-dead-volume valving and transfer of samples from one dimension to another is easily achieved through electroosmotic flow (EOF) supported by SU-8 [17]. In addition to direct coupling of a cIEF channel to on-chip ESI/MS, we also demonstrate a multiplex cIEF-transient isotachophoretic (tITP) chip design in which peptides are first separated based on cIEF (pH 3-10) and then isotachophoretically during EOF-driven mobilization through a subsequent separation channel to ESI/MS. The latter approach not only allows for effective preconcentration of the sample zones by cIEF, but also additional selectivity for peptide separations, as the migration order of the peptides is dissimilar from pure cIEF or ITP.

2. Materials and methods

2.1. Chemicals

All peptides (angiotensins I, II, and III, bradykinin (fragments 1-5), and substance P (fragment 6-11)), the amino acid histidine, ampholyte (pH 3-10), ammonium acetate, formic acid, Trizma base, and N, N, N´, N´-tetramethylethylenediamine (TEMED) were purchased from Sigma-Aldrich (Steinheim, Germany). Methanol, acetic acid, and ammonium hydroxide were from Mallinckrodt Baker B.V. (Deventer, The Netherlands) and acetonitrile was from Rathburn Chemicals Ltd. (Walkerburn, Scotland). Water was purified with a Milli-Q water purification system (Millipore, Molsheim, France) and all solutions were degassed by sonication for 10-15 min before use.
Negative photoresist SU-8 50 (Microchem, Newton, MA) used for chip fabrication was purchased from Microresist Technologies GmbH (Berlin, Germany) and poly(dimethyl siloxane) (PDMS, Sylgard 184, Dow Corning, Midland, MI) from VWR International Oy (Espoo, Finland).

2.2. Microchip Designs

The SU-8 microchips were fabricated by UV photolithography and adhesive bonding as described earlier [10, 12, 13, 16, 18]. Two types of microchip designs were used in the study. The first design, has been applied previously for MCE-ESI/MS [10], but was now used for direct coupling of cIEF to MS and comprised a 25-mm-long separation channel (50×50 μm^2, w×h) intersected by two 12-mm-long auxiliary channels (100×50 μm^2, w×h) providing the sheath liquid to an on-chip ESI emitter tip (Figure 1A). The second microchip design comprised two intersecting separation channels (both 50×50 μm^2, w×h), the first of which was used for cIEF (effective L=20 mm) prior to mobilization through the second channel (L=35 mm). This design was explicitly designed for this purpose. Here, one 12-mm-long auxiliary channel (150×50 μm^2, w×h) intersected with the mobilization channel just before the ESI emitter tip (Figure 1B). To compensate for potential peak broadening, the microchannel turns of the second chip design were tapered from the inner perimeter to a width of 30 µm. In addition, both designs also incorporated dummy side channels that facilitated easier filling. Before use, thin PDMS sheets with 2 mm inlet holes were attached on top of all chip inlets to delimit liquid spreading on the chip surface.

2.3. Direct coupling of microchip capillary isoelectric focusing to mass spectrometry

The cIEF-ESI/MS experiments were performed using the microchip design depicted in Figure 1A. The MS detection was performed on an ion trap 6330 MS (Agilent Technologies, Santa Clara, CA, USA) equipped with a CCD camera and an *xyz*-aligning stage for chip mounting. The MS was operated in positive ion mode with a capillary voltage of -1.6 kV, end-plate offset of -500 V, and a trap drive value of 94.6. Nitrogen (Nitrogen generator system, Parker, Cleveland, OH) was used as the drying gas (4.0 l min^{-1}, +70ºC) and data was acquired over a mass range of *m/z* 100-2200 with a maximum accumulation time of 100 ms. Data Analysis software was used for acquisition and data processing. An external µTK

power supply (Micralyne Inc., Edmonton, AB) was used for application of the cIEF, mobilization, and electrospray (ES) voltages through platinum wires placed in the microchannel inlets. Before analysis, the separation and sheath liquid channels were filled with sample solution and sheath liquid, respectively, by capillary flow. The sample solution contained the peptides dissolved in 2% ampholyte solution (pH 3-10) including 25% methanol and the sheath liquid was methanol:water 80:20 incorporating 1% acetic acid (Figure 1A). After filling, the sample solution in the sample inlet (SI) was replaced by the catholyte (1% ammonium hydroxide), whereas the sheath liquid also served as the anolyte during cIEF. Focusing of the peptides was performed at 2400 V cm^{-1} applied between BI and SLIs for 60 s, i.e., until an electrical current drop and stable reading was obtained. After focusing, the catholyte in the SI was replaced by the background electrolyte (BGE, 20 mM ammonium acetate with 25% methanol) and the peptides were mobilized toward the MS by applying electric field strength of 800 V/cm between the SI and the SLIs. During the mobilization step, the excess electrical current was grounded through a 50 MΩ resistor coupled in parallel with the ES voltage power supply similar to a previously described protocol [15].

2.4. Microchip capillary isoelectric focusing-transient isotachophoresis-mass spectrometry

The multiplex cIEF-tITP separations were performed using the microchip design depicted in Figure 1B. The MS experiments were performed on an API365 triple-quadrupole MS (Perkin-Elmer Sciex, Concord, ON, Canada) instrument equipped with an *xyz*-aligning stage and a CCD camera and operated in positive ion mode using nitrogen generated by a Whatman 75-720 nitrogen generator (Haverhill, MA) as the curtain gas. Data were recorded in full-scan MS mode with a dwell time of 350 ms per scan and a mass range of *m/z* 100-950. Analyst 1.4 software was used for acquisition and data processing. The high voltage (HV) instrumentation used for supplying the focusing, mobilization, and ES voltages was similar to that used in the cIEF-ESI/MS experiments. Before analysis, the separation channels and the auxiliary channel were filled with BGE (30 mM ammonium acetate with 30% methanol) and sheath liquid (methanol:water 80:20 with 1% acetic acid), respectively, by capillary flow and vacuum suction. After filling, the solution in the BI was replaced by BGE, the solution in the SI with sample solution (peptides in 2% ampholyte solution with 0.33% TEMED), and the solution in the SLI with sheath liquid. Inlet CA was filled with the catholyte (0.5-1% ammoniumhydroxide) and inlet AN with the anolyte (1% formic –or acetic acid) (Figure 1B). Then, the cIEF channel was loaded with the sample solution using electrokinetic flow at 500-1000 V cm^{-1} applied between reservoirs SI and BI for 100 s. Next, cIEF was performed by switching the potential difference (E=500-2000 V/cm) between the CA and the AN. Typically, a stable current reading was obtained in 200-270 s, which was considered sufficient for the formation of the pH gradient and subsequent focusing of the peptides based on their *pI* values. Last, the focused peptide zones were mobilized toward the MS by applying potential difference between the BI (6 kV) and the SLI (3.5 kV). The voltage applied to the SLI served not only as the counter voltage for the mobilization, but also as the ESI voltage (2.5 kV relative to MS). The electrical currents, recorded by multimeters and the μTK Editor software, were used for monitoring of the chip performance.

3. Results and discussion

In this study, two different chip designs were introduced to facilitate the online coupling of cIEF to on-chip ESI/MS detection. The first design (Figure 1A) was used for direct EOF-driven mobilization (of peptides focused by cIEF) to the MS *via* an integrated (two-sided) sheath flow interface. The second design (Figure 1B) incorporated two subsequent, intersecting separation channels which allowed us to perform peptide focusing by cIEF in the first dimension (effective L=20 mm) and then tITP separation during mobilization through the second dimension (L=35 mm). Also in this case, EOF-driven mobilization and an integrated (one-sided) ESI sheath flow interface were used. The performances of

the two microchip-based cIEF-ESI/MS setups were examined with help of peptide standards and also by monitoring the stability of the electrical currents during sample loading, focusing (cIEF), and mobilization.

3.1. Direct coupling of microchip capillary isoelectric focusing to mass spectrometry

The separation chip pictured in Figure 1A was used for direct coupling of cIEF to ESI/MS detection. It comprised a symmetrical (two-sided) sheath flow interface which allowed us to feed sheath liquid to the ESI tip from both sides of the separation channel. The organic sheath flow enhances the ES ionization process, especially when running buffers/samples contain high amounts of water. Here the sheath liquid also minimized major background interferences due to the carrier ampholytes and as a result, the carrier ampholytes were not observed when the cIEF pattern was mobilized to the MS (Figure 2). The acidic sheath liquid also served as the anolyte during cIEF so that the pH gradient (pH 3-10), along with the focused peptides, could be directly run to the MS after focusing. As a result, the model compounds, angiotensins I (*pI*=7.70) and II (*pI*=7.54) [19] migrated at about t_{migr}=23 s (Figure 2). Although the two angiotensins did not separate from each other because of their very similar *pI* values, they migrated as sharp, focused bands with peak capacity of about 86 indicating that efficient focusing takes place during the cIEF step.

This setup was somewhat similar to that previously published for protein separation by on-chip cIEF [20]. However, in our work, purely EOF-driven mobilization was used instead of hydrodynamic (pressure-driven) flow mobilization used in the previous work. Here, the possibility to use EOF-driven mobilization, and thus avoid the complex coupling to external pumps, was facilitated by the fact that our microchips were made of SU-8 polymer whose surface charge is inherently pH-dependent; basic buffers maintain cathodic- and acidic buffers anodic EOF [17]. Thus, the flow direction close to the anode (low pH) is opposing to that at the cathode (high pH) so that the net velocity in the SU-8-based channel during cIEF approaches zero. With most other microchip materials (or conventional silica capillaries), the cIEF separation channels need to be coated in order to eliminate the EOF. Alternatively, if not fully eliminated, the EOF should be significantly reduced to allow for complete focusing of the analytes before they migrate to the detector [21]. The advantage of having uncoated (charged) surfaces arise from the fact that, after focusing, EOF can be used for mobilization instead of pressure-driven flow. The pH-dependent surface charge of SU-8 was thus an excellent fit for the purpose so that EOF could be eliminated during cIEF and reverted for EOF-driven mobilization by simply switching the potential difference (Figure 1A) and replacing the catholyte in the SI with BGE.

Thanks to the relatively short separation channel, the cIEF focusing time in this work was also significantly shorter (60 s) compared to that applied (~10 min) in the previous work in which a 16-cm-long cIEF channel was used [20]. Of great importance is also the fact that the cIEF-ESI/MS experiments could be performed by using the same microchannel configuration as previously used for MCE-ESI/MS experiments by our group [10, 12, 13, 18]. This enables straightforward switching between microchip cIEF and MCE separation modes so that the best selectivity and resolving power are obtained for each type of sample.

3.2. Multiplex capillary isoelectric focusing and transient-isotachophoretic separation

Although feasible, direct coupling of microchip cIEF to ESI/MS is somewhat limited in terms of suitable solvents. Namely, only volatile acids with high organic content can be used as anolyte solution with setups similar to that presented in Figure 1A. Obviously, the resolving power of two compounds with very similar *pI* values (like angiotensins I and II) is also very challenging. Therefore, we developed a new two-dimensional separation chip in which cIEF separation (first dimension) was followed by tITP separation taking place in a subsequent separation channel (second dimension) during mobilization (Figure 1B). In this design, the anolyte composition is not limited to MS compatible solvents because this inlet is only active during cIEF focusing and the anolyte solution is never

mobilized toward ESI/MS detection. Thus, nonvolatile salts and aqueous acids/bases can also be used as anolytes, which often improves the stability of (the formation of) the pH gradient. However, filling of such a complex network of intersecting microchannels with multiple different solutions is somewhat challenging by using capillary flow. Therefore, we developed an electrokinetic sample loading protocol to facilitate straightforward filling of the chip with the ampholyte solution between the running buffer prior to cIEF-tITP separations.

Before analysis, all separation microchannels were filled with the same running buffer by capillary flow and vacuum suction. The sample in 2% ampholyte solution was then loaded only into the cIEF part (SI↔BI) using electrokinetic flow as described in Figure 1B (i.e., loading step). In order to understand and monitor the electrokinetic migration taking place, the electrical current was monitored during the loading process as well as during focusing and mobilization steps (Figure 3). A closer examination of the current profile during the loading step suggests that, in addition to filling of the cIEF channel, tIEF takes place already during the loading step. Namely, during the first 25 s, the total amount of charges decrease indicating filling of the cIEF channel with ampholyte solution and simultaneous formation of the pH gradient by the carrier ampholytes in the sample plug entering the cIEF channel. As a result, the sample components and the carrier ampholytes start to arrange according to their *pI* already during the loading step which allows for relatively short focusing times in the following cIEF step. However, a subsequent current increase is observed between 45 and 55 s (Figure 3), which indicates progressive migration of the acetate counter-ions from the BI into the cIEF channel. This in turn results in drift in the pH gradient and slow fluctuation of the electrical current between 50 and 100 s (Figure 3), similar to earlier work performed with conventional CE capillaries [22]. Therefore, the sample components (or the carrier ampholytes) do not readily reach their true isoelectric points during the loading step, but are stacked isotachophoretically based on both their *pI* values and intrinsic electrophoretic mobilities (μ_{EP}) [22, 23]. Thus, the cIEF arrangement must be finalized during the second focusing step (Figure 3) by switching the potential difference between the anolyte (AN) and the catholyte (CA). Here, 1% formic acid or acetic acid was used as the anolyte while the catholyte was 0.5-1% ammoniumhydroxide. These solutions provide opposing EOF and thus zero net velocity between inlets AN and CA similar to that described for the one-dimensional chip design. Further examination of the current profile during the focusing step (Figure 3) indicates formation of a more stable pH gradient within about 60 s as evidenced by the continuous current drop with a steady state current levelling off at around 15 µA (Figure 3). The repeatability of the steady state current between repeated runs (n=6) was 8.9% RSD.

Once focusing is complete, the pH gradient is mobilized toward the MS using electrokinetic flow similar to the one-dimensional design, i.e., by switching potential difference between BI and the SLI. However, in this case, the sample plug (i.e., the pH gradient) is preceded and followed by a relatively long plug of the running buffer which acts as a leading electrolyte (LE). Accordingly, the ampholyte solution acts as the terminating electrolyte (TE) sandwitched between two LE zones similar to tITP [22]. As a result, instead of just mobilizing a pure cIEF gradient toward the MS, the separation mode during the mobilization step is more likely tITP so that the observed migration order is affected by both the *pI* value and the intrinsic electrophoretic mobility of the sample component. After cIEF focusing the back of the ampholyte (TE) plug starts to dissolve into the running buffer (LE) because the faster BGE ions overcome the ampholyte ions. As a result, the analyte ions migrate (according to their μ_{EP}) and focus at the front interface between the ampholyte (TE) and the running buffer (LE). Finally, when all ampholyte ions are dissolved, the analyte ions are separated according to the principles of zone electrophoresis. The *pI* values mainly determine the position of the sample components in the cIEF channel, and thus their (individual) effective separation length from the cIEF channel to the ESI tip. The intrinsic electrophoretic mobilities (in the ampholyte solution), in turn, determine the speed in which each sample component is migrating toward the MS and the resulting migration order is dissimilar from both pure cIEF and pure ITP. This is clearly illustrated with the separation of histidine

(*pI*=7.55) [19] and angiotensin I (*pI*=7.70) [19] in Figure 3. Although the *pI* values are very close to each other (ΔpI=0.15), these two compounds are baseline-resolved (R_S=1.9) thanks to their very different size and thus, different intrinsic electrophoretic mobilities.

In this case, the electrophoretic mobility is not affecting the migration order of histidine and angiotensin I, which migrate in order of increasing *pI*, as expected. Instead, the effect of the electrophoretic mobility on the migration order becomes more prominent, when analyzing a mixture of five peptides with greater distribution in the *pI* values, namely bradykinin (fragment 1-5, *pI*=10.55), angiotensin III (*pI*=9.35), substance P (fragment 6-11, *pI*=7.81), angiotensin I (*pI*=7.70), and angiotensin II (*pI*=7.54) [19]. In order to prevent the most basic pH range from shifting in a distal region of the microchannel (i.e., beyond the Y intersection close to inlet CA), TEMED was added to a final concentration of 0.33% (v/v) in the 2% ampholyte solution [24, 25]. As a result, the theoretical position of pH 10 was 3.3 mm downstream from the Y intersection. Under these conditions, the most basic peptide, bradykinin (*pI*=10.55), migrates and focuses to the interface of TEMED and the ampholyte solution (pH 10) after which it is diffused into the TEMED zone (Figure 4). The theoretical position of the other peptides after the focusing step is given according to their *pI* values in Figure 4. However, now, the observed migration order of the five model peptides does not follow the order of increasing *pI*, but is largely affected by the peptides' intrinsic electrophoretic mobilities. For instance, angiotensin III and substance P (fragment 6-11), which both show relatively high cathodic electrophoretic mobilities, are able to bypass angiotensins I and II (featuring slow anodic mobilities in the ampholyte solution) during the mobilization step (Figure 4). Instead, bradykinin, which also shows high cathodic mobility, but is trapped in the TEMED zone based on its *pI* value, will not catch the angiotensins I and II and migrates last. Therefore, the coupling of cIEF separation with electrokinetic mobilization (in tITP mode) as presented in this study provides different kind of selectivity for the separation than tITP alone. Although the migration order of such multiplex cIEF-tITP separation is difficult to predict theoretically, it was shown to be very well repeatable in terms of the migration times that were within 3.1-6.8% RSD (n=3) for all of the peptides.

A closer examination of the bradykinin peak shape also evidences that cIEF arrangement has indeed taken place during the focusing step: the sharp front of the bradykinin peak indicates focusing to the borderline of TEMED (based on *pI*), whereas the tailing peak is due to diffusion into the TEMED zone (during cIEF once the electrophoretic mobility has ceased). Comparison of the peptides' intrinsic electrophoretic mobilities in the plain BGE and in the 2% ampholyte solution also reveals that addition of ampholytes into the running buffer not only has a clear impact on the resulting EOF (2.5×10^{-4} cm^2 (Vs)$^{-1}$ $\rightarrow$ 1.4×10^{-4} cm^2 (Vs)$^{-1}$), but also on the electrophoretic mobilities. This is particularly the case of angiotensins I and II, which shift from cathodic to anodic mobility. As a result, three peptides with nearly identical *pI* (i.e., angiotensin II, *pI*=7.54; angiotensin I, *pI*=7.70; substance P, fragment 6-11, *pI*=7.81 [19]) are efficiently resolved in multiplex cIEF-tITP mode (Figure 4).

4. Conclusions

In this work, we have addressed the challenges of the combination of microchip cIEF with online, on-chip ESI/MS detection by coupling of the two *via* an immediate on-chip sheath flow ESI interface. The robustness of the developed setup was shown by coupling the chips to two different types of mass analyzers, namely an iontrap and a triple quadrupole mass spectrometer. The pH-dependent surface charge of the SU-8 polymer used for microchip fabrication enabled EOF-driven mobilization (high pH) on one hand and elimination of EOF (low pH) for successful cIEF in native channels without any surface pretreatment on the other hand. In all, our results clearly emphasize the potential of microchip-based cIEF-ESI/MS in peptide analysis. In addition to direct coupling of cIEF separation to on-chip ESI/MS, parallel microfabrication technology applied herein facilitates construction of relatively

complex, yet dead-volume-free fluidic networks featuring multiple intersecting microchannels that can be exploited to multiplex separations not feasible for conventional capillary systems, like the combination of cIEF-tITP demonstrated in this work. An obvious challenge of the multiplex cIEF-tITP approach presented is the fact that the theoretical migration order is difficult to predict based on pure cIEF or pure ITP separation rules. This is, however, not a limiting factor in case the microchip setup is combined with MS detection capable of identifying the sample components based on their mass-to-charge ratios. In return, additional selectivity and increased resolving power can be obtained compared to pure cIEF or pure ITP (or zone electrophoresis) separation modes alone. To our knowledge, there are very few prior reports on the combination of cIEF and ITP on chip or on the microchip cIEF-MS coupling in general.

Acknowledgements
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5. References

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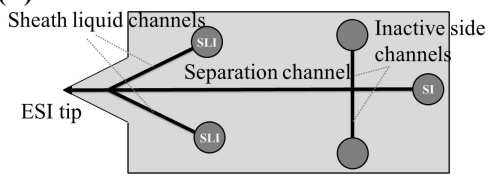
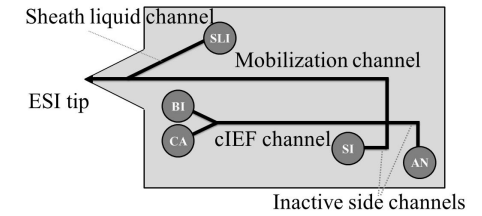
(A)



Steps	SI	SLI	SLI
Focusing (60 s)	Ground	6.0 kV	6.0 kV
Mobilization	4.0 kV	2.0 kV	2.0 kV

(B)



Steps	BI	CA	SI	AN	SLI
Loading (100-120 s)	Ground	Float	1.5-3 kV	Float	Float
cIEF (200-270 s)	Float	Ground	Float	1.5-6 kV	Float
Mobilization	6 kV	Float	Float	Float	3.5 kV

Figure 1. SU-8-based microchips. Schematic views of the SU-8 microchips used (A) for direct coupling of cIEF to on-chip ESI/MS and (B) for multiplex cIEF-tITP separation coupled to on-chip ESI/MS together with high voltage sequences used. In (A), filling of the separation channel with the sample solution was done by capillary flow from the sample inlet (SI), after which the focusing potential was applied between the sheath liquid inlets (SLI, anolyte/sheath liquid) and the SI (catholyte). Mobilization of the peptides toward the MS was done by changing the solution in SI to the background electrolyte (BGE) and switching on the mobilization voltages. In (B), loading, cIEF, and mobilization were performed electrokinetically with the voltages given in the table. BI=buffer inelt. SI=sample inlet. CA=catholyte. AN=anolyte. SLI=sheath liquid inlet.

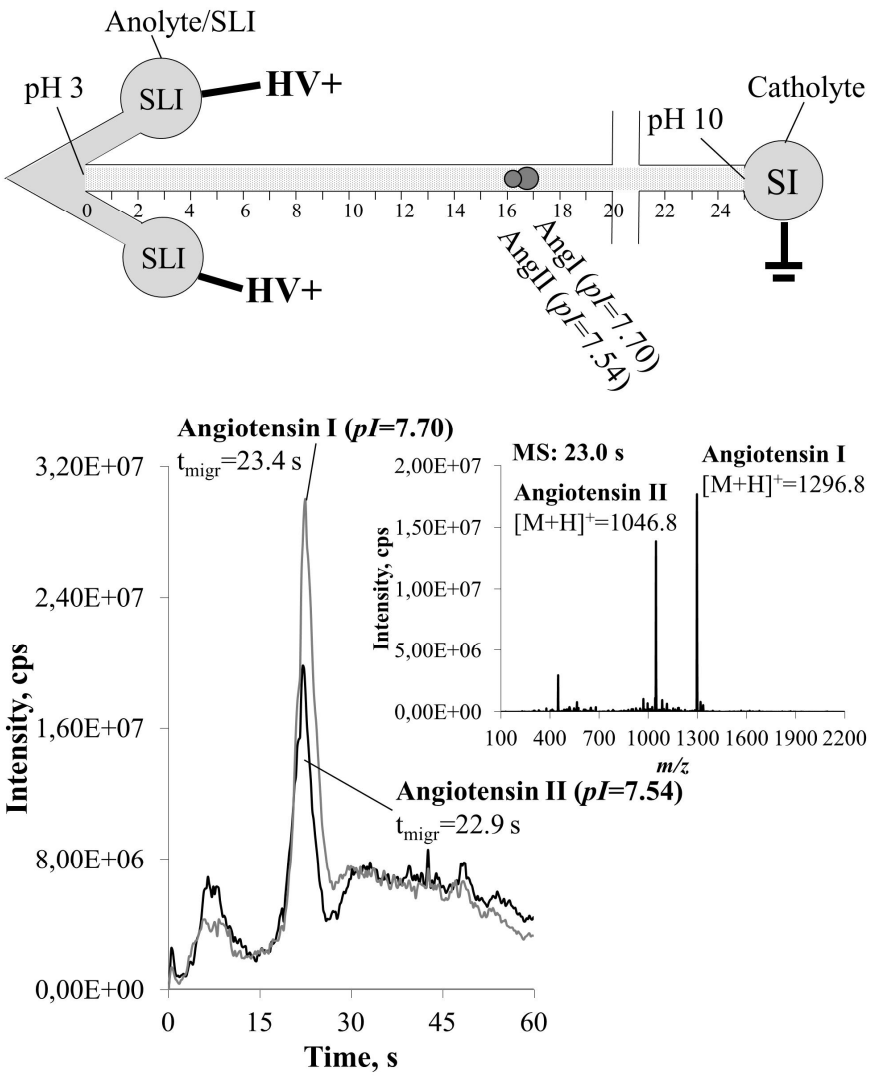


Figure 2. Microchip cIEF-ESI/MS of angiotensins I and II. Schematic illustration of the theoretical position of angiotensin I (AngI) and II (AngII) after cIEF separation and extracted ion electropherograms (EIE) of the same peptides (both 0.2 mg mL^{-1}) following cIEF separation, mobilization, and ESI/MS detection performed using the chip design depicted in Figure 1A. The size of the circles in the schematic illustration assimilates the molecular masses of the peptides. Time t=0 s in the EIE corresponds to the end of the focusing step and start of the mobilization step. The focusing was performed in 2% ampholyte solution (pH 3-10) including 25% methanol at 2400 V cm^{-1} for 60 s. The mobilization was done by feeding BGE (20 mM ammonium acetate with 25% methanol) from the SI at E=800 V cm^{-1}. The mass spectrum recorded at t=23 s (insert on the right, no background subtraktion) shows abundant protonated ions of AngI and AngII and negligible background interference due to the carrier ampholytes.

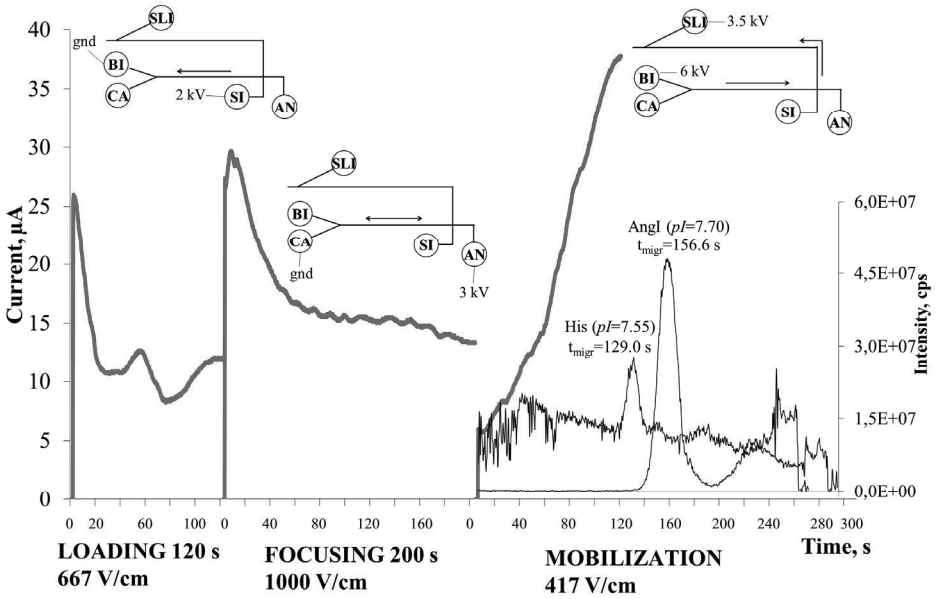


Figure 3. Electrical current profiles. The electrical current profiles (average from six repeated runs) during sample loading (SI→BI), focusing (AN→CA), and mobilization (BI→SLI) along with the EIEs of histidine (His, 100 µM, $[M+H]^{+}$=156.0) and angiotensin I (AngI, 100 µM, $[M+2H]^{2+}$=649.8) separated by cIEF-tITP-ESI/MS. The anolyte (AN) was 1% acetic acid, the catholyte (CA) 1% ammoniumhydroxide, the BGE (BI) 30 mM ammonium acetate with 30% methanol, and the sheath liquid (SLI) methanol:water 80:20 with 1% acetic acid.

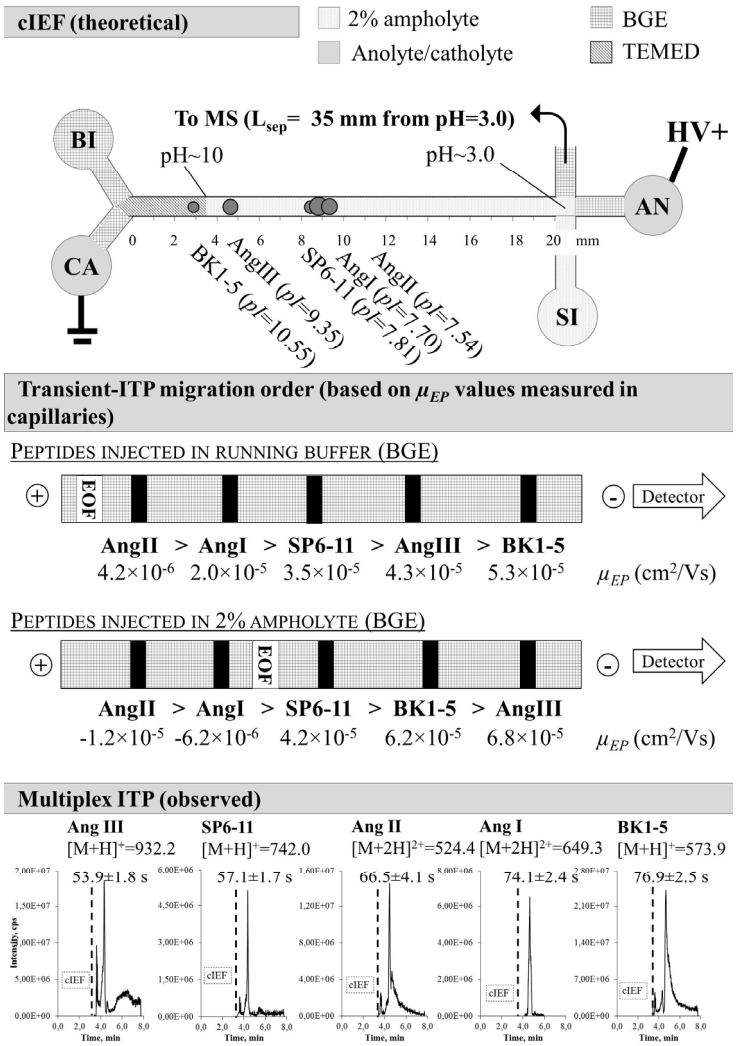


Figure 4. Multiplex cIEF-tITP-ESI/MS of peptide standards. Schematic illustration of the theoretical position of bradykinin fragment 1-5 (BK1-5), angiotensin III (AngIII), substance P fragment 6-11 (SP6-11), angiotensin I (AngI), and angiotensin II (AngII) following cIEF. The size of the circles assimilates the molecular masses of the peptides. The theoretical ITP migration order is given based on the intrinsic electrophoretic mobilities (μ_{EP}) measured in conventional silica capillaries by injecting peptides in the running buffer or in the 2% ampholyte solution (see Supplementary data). The extracted ion electropherograms (EIE) of the same peptides from multiplex cIEF-tITP analysis, performed with the two-dimensional chip, design show both the focusing (200 s at 1333 V cm^{-1}) and mobilization (417 V cm^{-1}) steps. Loading of the cIEF channel with the sample solution was performed at 1000 V cm^{-1} for 100 s. The sample solution contained peptides (each 100 μM), 2% ampholyte (pH 3-10), and 0.33% TEMED. The anolyte was 1% formic acid, the catholyte was 0.5% ammoniumhydroxide while the running buffer and the sheath liquid were according to Figure 3. The EIE of angiotensin I has been background subtracted with respect to the background ions *m/z*=648.0 and *m/z*=650.2. Time t=0 s in the electropherograms corresponds to the end of the focusing step (also indicated with dashed line).

Supplementary data

Interfacing Microchip Isoelectric Focusing with On-chip Electrospray Ionization Mass Spectrometry

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Keywords: microchip electrophoresis, isoelectric focusing, isotachophoresis, mass spectrometry, microfluidics

Determination of electrophoretic mobilities (μ_{EP})

Materials and methods

Chemicals

Coumarin used as an electroosmotic flow (EOF) marker was purchased from Sigma Aldrich (Steinheim, Germany). All other chemicals were the same as those reported in the Materials and methods section in the main paper.

Instrumentation

The capillary electrophoresis (CE) instrumentation used for determination of the electrophoretic mobilities (μ_{EP}) of the peptides was a P/ACE MDQ CE equipment (Beckman Instruments, CA, USA) equipped with a UV detector (λ_{abs}= 214 nm) and temperature control units for the capillary and samples. The fused silica capillary (I.D. 50 µm, O.D. 375 µm, length 47 cm) was purchased from Composite Metal Services (Werchester, UK). Hydrodynamic injections (15 s, 0.5 psi (3.4 kPa)) were performed in duplicate. The effective separation length was 40 cm and the separation voltage applied was 25 kV (532 V cm^{-1}). The background electrolyte (BGE) was 30 mM ammonium acetate with 30% methanol. Before use the capillary was conditioned sequentially with 0.1 M NaOH containing 30% methanol, water:methanol 70:30 (v/v), and BGE by flushing at 20 psi (137.9 kPa) for 15 min each. Between analyses the capillary was flushed with BGE at 20 psi for 2 min.

Calculation of the electrophoretic mobilities (μ_{EP})

The μ_{EP} of a component, *i*, in an electric field can be expressed by Equation (S1):

$$\mu_{EP,i} = \mu_{app,i} - \mu_{EOF} = \frac{L_D}{t_i \cdot E} - \frac{L_D}{t_{EOF} \cdot E} = \frac{L_D}{E}\left(\frac{1}{t_i} - \frac{1}{t_{EOF}}\right) \quad \text{(S1)}$$

where μ_{app} is the net mobility of component *i* [cm^2 (Vs)$^{-1}$], μ_{EOF} is the mobility of the electroosmotic flow [cm^2/Vs], L_D is the effective separation length [cm], t_i is the migration time of component *i* [s], t_{EOF} is the migration time of the EOF marker, coumarin [s], and E is the electric field strength [V cm^{-1}]. The μ_{EP}:s of the five peptide standards used were determined by injecting them in the BGE (30 mM ammonium acetate with 30% methanol) or in 2% ampholyte solution (pH range 3-10) including 0.33% N, N, N´, N´-tetramethylethylenediamine (TEMED) (Table S1). The running buffer was the same in both cases (i.e., the BGE, 30 mM ammonium acetate with 30% methanol).

Table S1. The electrophoretic mobilities (μ_{EP}) of the five model peptides used in the study as determined by conventional CE-UV instrument.

Peptide	***pI****	**μ_{EP} in BGE [cm^2 (Vs)$^{-1}$]**	**μ_{EP} in ampholyte [cm^2 (Vs)$^{-1}$]**
Bradykinin 1-5	10.55	5.27×10^{-5}	6.19×10^{-5}
Angiotensin III	9.35	4.27×10^{-5}	6.76×10^{-5}
Substance P 6-11	7.81	3.53×10^{-5}	4.21×10^{-5}
Angiotensin I	7.70	1.98×10^{-5}	-6.16×10^{-6}
Angiotensin II	7.54	4.15×10^{-6}	-1.24×10^{-5}

*derived from GenScript Peptide Property Calculator (*https://www.genscript.com/ssl-bin/site2/peptide_calculation.cgi*)

III

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Rapid and sensitive drug metabolism studies by SU-8 microchip capillary electrophoresis-electrospray ionization mass spectrometry

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ABSTRACT

Monolithically integrated, polymer (SU-8) microchips comprising an electrophoretic separation unit, a sheath flow interface, and an electrospray ionization (ESI) emitter were developed to improve the speed and throughput of metabolism research. Validation of the microchip method was performed using bufuralol 1-hydroxylation *via* CYP450 enzymes as the model reaction. The metabolite, 1-hydroxybufuralol, was easily separated from the substrate (R_s = 0.5) with very good detection sensitivity (LOD = 9.3 nM), linearity (range: 50–500 nM, r^2 = 0.9997), and repeatability (RSD_{Area} = 10.3%, $RSD_{Migration\ time}$ = 2.5% at 80 nM concentration without internal standard). The kinetic parameters of bufuralol 1-hydroxylation determined by the microchip capillary electrophoresis (CE)-ESI/mass spectrometry (MS) method, were comparable to the values presented in literature as well as to the values determined by in-house liquid chromatography (LC)-UV. In addition to enzyme kinetics, metabolic profiling was demonstrated using authentic urine samples from healthy volunteers after intake of either tramadol or paracetamol. As a result, six metabolites of tramadol and four metabolites of paracetamol, including both phase I oxidation products and phase II conjugation products, were detected and separated from each other within 30–35 s. Before analysis, the urine samples were pre-treated with on-chip, on-line liquid-phase microextraction (LPME) and the results were compared to those obtained from urine samples pre-treated with conventional C18 solid-phase extraction (SPE, off-chip cartridges). On the basis of our results, the SU-8 CE-ESI/MS microchips incorporating on-chip sample pre-treatment, injection, separation, and ESI/MS detection were proven as efficient and versatile tools for drug metabolism research.

1. Introduction

One of the major reasons for the termination of the development of potential drug candidates is their poor adsorption, distribution, metabolism, and excretion (ADME) properties. To prevent expensive terminations in late clinical stages, much effort needs to be expended to investigate the ADME characteristics as early in the drug discovery process as possible [1]. Metabolic profiling of new drug candidates is particularly important and includes not only identification of metabolites, but also screening of their properties, such as stability and toxicity [2]. Also screening of drug–drug interactions and determining the kinetic parameters of the drug metabolism are equally important [2]. In these types of analyses, the key issue is high throughput and therefore the analytical method should be as fast as possible. Sometimes the amounts of the metabolites are extremely low and thus, highly specific and sensitive analytical methods are also required.

Presently, most metabolism assays are performed by gas (GC) – or liquid (LC) chromatography combined with mass spectrometry (MS) or by nuclear magnetic resonance (NMR) spectroscopy [3,4]. Since a vast majority of metabolites are polar and ionic, capillary electrophoresis (CE) is another approach for the separation of metabolites, in addition to LC analysis. Microchip CE in particular provides significant improvement in terms of fast analysis times and enables direct coupling to electrospray ionization (ESI)/MS which eventually results in high sensitivity and good selectivity. Although microchip CE with electrochemical [5,6] or optical [7–9] detection has occasionally been applied to metabolism research, the use of MS detection is still rare. Even though most microchip methods provide considerable increase regarding speed of analysis, the time-consuming (off-chip) sample pre-treatment often prolongs the total analysis time. Typically, complex biological matrices,

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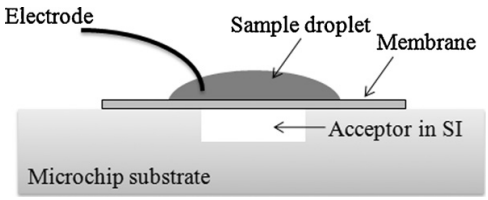


Fig. 1. Schematic illustration of the on-chip droplet-membrane-droplet LPME system on top of an SU-8 CE-ESI/MS microchip. The sample solution was alkaline (30 mM NaOH, pH 11.4) urine. The acidic (0.1% formic acid, pH 2.7) acceptor solution contained 100 μM verapamil as an internal qualifier. SI = sample inlet.

such as urine, require extensive and laborious clean-up before analysis. Thus, integration of sample pretreatment with injection, separation and detection on a single microchip increases sample throughput and approaches the μTAS (micro total analysis systems) concept [10].

Today, modern lithographic and adhesive bonding techniques enable mass production of very complex and accurately defined microstructures on highly integrated devices [11]. Recently, the epoxy-based negative photoresist SU-8 has shown to be a very suitable material for microchip production [12–15]. It is easily patterned by standard photolithography, it has excellent thermal and mechanical properties, and it is also stable against many acids, bases and solvents. In this work, highly integrated CE-ESI/MS microchips were fabricated from SU-8 polymer so that all critical structures were simultaneously patterned by photolithography. SU-8 microfabrication technology relies on photolithography and wafer-level bonding, an approach that enables production of tens of identical chips on one wafer [12,16]. Also reproducibility from wafer-to-wafer and batch-to-batch is very good [17]. Here, the applicability of the microchips to metabolism research was first demonstrated by determining the kinetic parameters of the cytochrome P450 (CYP) mediated bufuralol 1-hydroxylation by microchip CE-ESI/tandem MS (MS/MS). In addition, authentic urine samples were screened for metabolites of tramadol and paracetamol by microchip CE-ESI/MS after on-chip liquid-phase microextraction (LPME). To the best of our knowledge, CE-ESI/MS microchips have not been previously used in drug metabolism research, which is highly demanding because of the need for very low detection limits and highly reproducible and quantitative determination of the produced metabolites. Instead, most of the previous work has gone into development of microchips for qualitative protein analysis [18–20].

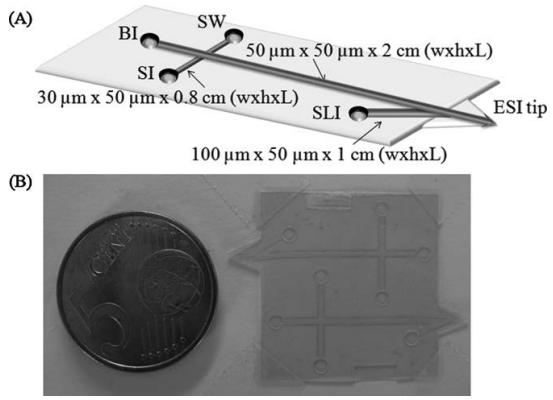


Fig. 2. (A) Schematic view of the fluidic design of the SU-8 CE-ESI/MS microchip (dimensions not to scale) and (B) photograph of the SU-8 microchip. BI = buffer inlet, SI = sample inlet, SW = sample waste, SLI = sheath liquid inlet.

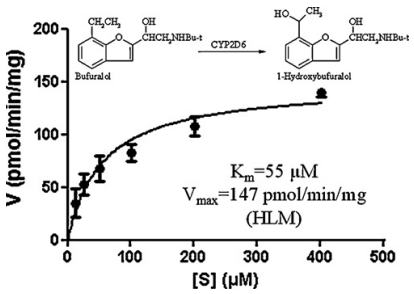


Fig. 3. Michaelis–Menten kinetics of the CYP450 mediated bufuralol metabolism to 1-hydroxybufuralol in HLM determined by microchip CE-ESI/MS in SRM mode. The separation was performed under electric field strength of 750 V/cm in 30 mM ammonium acetate with 50% methanol as BGE and 80% methanol–20% water with 1% acetic acid as sheath liquid. The substrate concentration ranged from 12.5 to 400 μM and all incubations were done in duplicate at each substrate concentration.

2. Materials and methods

2.1. Chemicals

Bufuralol was obtained from Roche (Basel, Switzerland), 1-hydroxy bufuralol was from Ultrafine Chemicals (Manchester, England), paracetamol was from Orion Pharma (Espoo, Finland), paracetamol glucuronide and verapamil hydrochloride were from Sigma–Aldrich (Steinheim, Germany), and tramadol as well as the metabolites *O*-desmethyltramadol (M1), *N*,*O*-didesmethyltramadol (M5) were kindly donated by the Department of Forensic Medicine, University of Helsinki, Finland. Human liver microsomes (HLM) were purchased from BD Gentest™ (Erembodegem, Belgium) and nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) was from Sigma–Aldrich. Acetic acid, formic acid, ammonium formate and 1-methoxy-2-propyl acetate (PMA) were all purchased from Sigma–Aldrich, sodium hydroxide and methanol were from J.T. Baker (Deventer, Holland), acetonitrile was from VWR (Espoo, Finland), hydrochloride and sodium phosphate from Riedel de Haen (Seelze, Germany), and 1-octanol and ammonium acetate were from Fluka (Buchs, Switzerland). Water was purified with a Milli-Q water purification system (Millipore, Molsheim, France).

2.2. Enzyme incubations

The kinetic parameters of the bufuralol 1-hydroxylation in HLM were determined by monitoring the CYP mediated reaction during incubation at 37 °C for 60 min. The incubation conditions were optimized in-house (data not shown) and six different substrate concentrations were used, i.e., 12.5, 25, 50, 100, 200 and 400 μM (two replicates of each). The incubation mixture (100 μL) contained bufuralol (12.5–400 μM), 50 mM sodium phosphate buffer (pH 7.4), HLM (0.8 mg/mL) and NADPH (1 mM), and the reaction was terminated by the addition of 100 μL of ice-cold acetonitrile. After removal of the proteins by centrifugation (5 min, 13,000 rpm), the supernatant was analyzed without further treatment. Blank samples were prepared without NADPH, HLM or substrate as well as with zero incubation time. GraphPad Prism 5.01 was used for data processing.

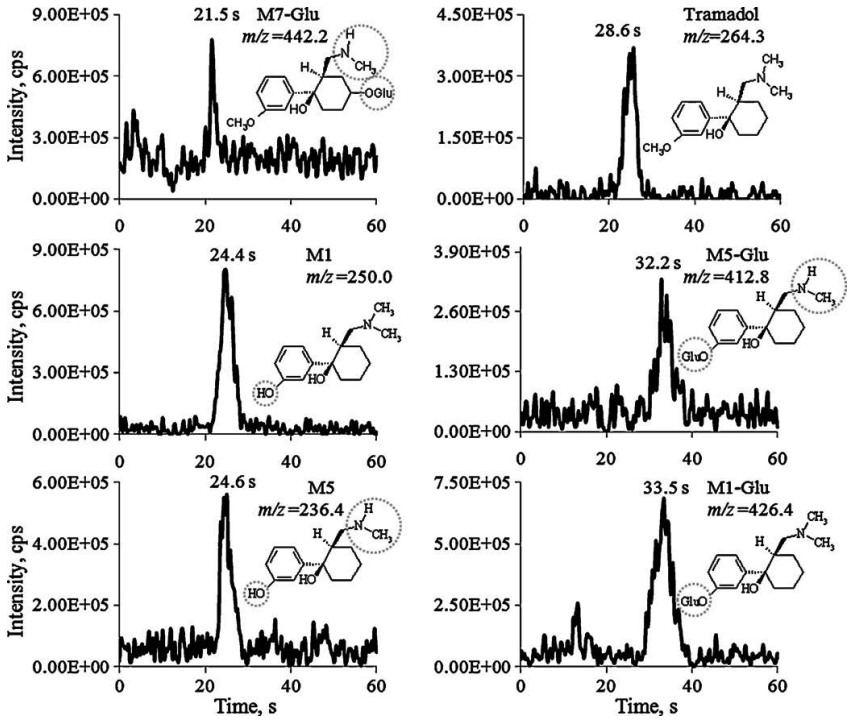


Fig. 4. Extracted ion electropherograms (EIE) of tramadol and its metabolites detected from a human urine sample and separated by microchip CE-ESI/MS after SPE (5-fold preconcentration). The BGE was 30 mM ammonium acetate with 50% methanol and the sheath liquid was 80% methanol–20% water with 1% acetic acid. The electric field strengths during injection (20 s) and separation were 1000 and 800 V/cm, respectively.

2.3. Urine samples

Urine samples were collected from two healthy volunteers 4 h after paracetamol (500 mg) intake or 12 h after tramadol (50 mg) intake. Urine samples were stored frozen at −20 °C until use and pretreated either by off-chip solid-phase extraction (SPE) or by on-chip LPME before analysis.

2.4. Off-chip solid phase extraction

Two different SPE extraction sorbents were used for the urine samples containing paracetamol or tramadol metabolites. For paracetamol samples, an Isolute MF C18, 100 mg reversed phase cartridge (International Sorbent Technology Ltd., Mid Glamorgan, U.K.) was conditioned with 1 mL methanol and balanced with 1 mL of 50 mM HCl in 2% methanol. One milliliter of paracetamol urine was acidified with 50 μL of 1 M HCl and slowly loaded onto the cartridge and washed with 1 mL 10 mM HCl. The analytes were eluted with 1.5 mL of methanol. For tramadol urine samples an Oasis HBL, 30 mg cartridge (Waters, Milford, MA, USA) was conditioned with 1 mL of methanol and 1 mL of water. The tramadol urine sample (1 mL) was slowly loaded onto the cartridge and washed with 1 mL of water. Elution was performed with 1 mL of methanol. The extracts of both the tramadol and paracetamol urine were evaporated to dryness under nitrogen and the residues were reconstituted in 200 μL of 10 mM ammonium acetate containing 50% methanol (5-fold concentration).

2.5. On-chip liquid-phase-microextraction

On-chip LPME was performed as previously described [21] and as illustrated in Fig. 1. An aliquot of 2 μL of 0.1% formic acid (pH 2.7) with 100 μM verapamil as an internal qualifier (acceptor solution) was applied to the sample inlet (SI) of the SU-8 CE-ESI/MS microchip (Fig. 2). A 5 mm × 5 mm piece of a Celgard 2500 microporous polypropylene membrane (Celgard, Charlotte, NC, USA) with a 25 μm thickness, 55% porosity, and 0.21 μm × 0.05 μm pores was wetted with 1-octanol and placed on top of the acceptor solution. Finally, 4 μL of alkaline tramadol urine sample (30 mM sodium hydroxide, pH 11.4, donor solution) was applied on top of the membrane to initiate extraction. After 5 min, the polypropylene membrane was removed and a platinum electrode was placed in the acceptor solution in the SI. The injection voltages were immediately applied and the injection was performed in pinched mode for 60 s before application of the CE separation voltages.

2.6. Microchip capillary electrophoresis

The microchips comprising a monolithically integrated injection and separation unit, a sheath flow interface and an ESI emitter were fabricated entirely of epoxy photoresist SU-8 using photolithography and adhesive bonding techniques as reported earlier [12,13]. The effective separation length of the CE chip was 2 cm. Other microchannel dimensions are given in Fig. 2. Before use, poly(dimethylsiloxane) (PDMS) sheets with 2 mm inlet holes were attached on top of the SU-8 chips to increase the inlet volumes. On the microchips that were used for the LPME experiments, the SI (Fig. 2) was left uncovered.

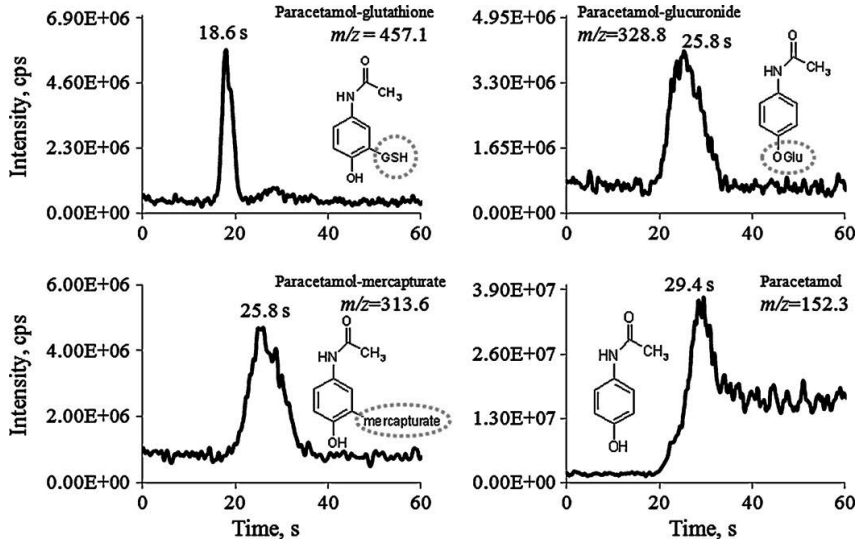
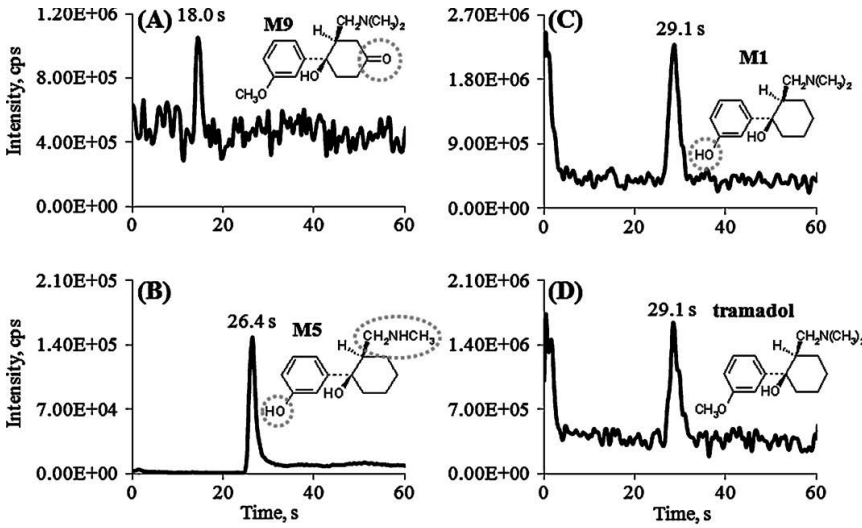


Fig. 5. Extracted ion electropherograms (EIE) of paracetamol and its metabolites detected from a human urine sample and separated by microchip CE-ESI/MS after SPE (5-fold preconcentration). The BGE was 30 mM ammonium acetate with 50% methanol and the sheath liquid was 80% methanol–20% water with 1% acetic acid. The electric field strengths during injection (20 s) and separation were 1000 and 500 V/cm, respectively.



Metabolite		m/z	MS mode*	pKa		logD	
				Amine	Phenol	pH 11	pH 3
(A)	M9	278.6	EIE	9.28	-	1.26	-2.23
(B)	M5	236.1→44.2	SRM	10.02	9.22	0.34	-1.32
(C)	M1	250.5	EIE	8.97	9.62	0.74	-1.20
(D)	tramadol	264.3	EIE	9.23	-	2.44	-1.05

*EIE=extracted ion electropherogram, SRM=selected reaction monitoring

Fig. 6. Extracted ion electropherograms (EIE, M9, M1 and tramadol) and selected reaction monitoring (SRM) electropherogram (M5) of tramadol and its metabolites detected from a human urine sample and separated by microchip CE-ESI/MS or MS/MS after on-chip LPME (2-fold preconcentration). The LPME was performed from alkaline urine (NaOH 30 mM, pH 11.4) into acidic acceptor solution (0.1% formic acid, pH 2.7) for 5 min followed by injection (60 s, 1000 V/cm) and separation (750 V/cm) in 30 mM ammonium formate with 50% methanol. The sheath liquid consisted of 80% methanol–20% water with 1% formic acid. Log D and pK_a values were derived from the online MarvinSketch chemical editor at www.chemaxon.com.

The samples were injected electrokinetically (20–60 s) in pinched injection mode with an electric field strength of 1000 V/cm applied between the SI and the sample waste (SW). Simultaneously, a small focusing potential was applied to the buffer inlet (BI) to avoid sample leakage into the separation channel (Fig. 2). The sheath liquid inlet (SLI) was left floating during injection so that no spray was produced. The CE separations were performed in cathodic mode using electric field strengths of 500–800 V/cm between the BI and SLI. In addition, small push-back voltages were applied to the SI and SW. The background electrolyte (BGE) consisted of 30 mM ammonium acetate (pH 7.0) or ammonium formate (pH 6.4) with 50% methanol, while the sheath liquid was methanol:water 80:20 (v/v) with 1% acetic or formic acid, respectively.

2.7. Mass spectrometry

The microchips were placed on an *xyz*-aligning stage in front of an API3000 or an API365 triple–quadrupole MS (Perkin-Elmer Sciex, Concord, ON, Canada). An external power supply (Micralyne Inc., Edmonton, Canada) was used for application of the injection, separation and electrospray (ES) voltages through platinum wires placed in the liquid filled inlets. The MS was operated in positive ion mode with an ES voltage of 3.6 kV (relative to MS) applied through the SLI. This voltage also served as the counter voltage for the CE separation. The separation current was divided at the sheath flow intersection into the ES and the auxiliary channel from where the excess current was led to ground through a 50 MΩ resistor coupled in parallel with the ES voltage power supply. Data were recorded in full-scan MS mode (metabolic profiling) with a dwell time of 300 ms per scan and a mass range of m/z 50–500 or in selected reaction monitoring (SRM) mode (enzyme kinetics) with a dwell time of 50 ms per selected precursor/product ion pair. Analyst 1.4 software was used for data acquisition and processing.

2.8. High performance liquid chromatography (a reference method)

In addition to microchip CE-ESI/MS, the kinetic parameters of the bufuralol 1-hydroxylation were determined based on conventional high performance liquid chromatography (HPLC)-UV analysis. The HPLC instrument was an Agilent 1100 (Agilent Technologies, Palo Alto, CA, USA) equipped with an autosampler and a UV multiple wavelength detector. The HPLC separation was performed by reversed phase chromatography using a Zorbax Eclipse Plus C18 column (5 μm, 150 mm × 4.6 mm, Agilent Technologies, Palo Alto, CA, USA). The injection volume was 20 μL and the eluent consisted of 0.1% formic acid (A) and methanol (B) with a gradient profile from 20% to 90% B in 6 min followed by 90% B for 5 min. The flow rate was 1 mL/min and the UV detection wavelength was 250 nm.

3. Results and discussion

The performance of the SU-8 microchips in quantitative CE-ESI/MS analysis was validated using 1-hydroxy bufuralol as a standard. The limit of detection (LOD, S/N = 3) and limit of quantitation (LOQ, S/N = 10), as per the ICH guidelines, were 9.3 and 31.2 nM, respectively. This corresponds to sample amounts of only 0.42 and 1.4 attomol for LOD and LOQ, respectively, as per injection volume of 45 pL (determined by the volume of the intersection of the separation and the injection channels). The regression coefficient (r^2) in the concentration range 50–500 nM was 0.9997 indicating good linearity. At best, the relative standard deviations (RSD, at 80 nM, $n = 5$) of the peak area and the migration time were 10.3% and 2.5%, respectively, which indicated relatively good quantitative performance of the microchip-based system even without the use of an internal standard. All values were determined using SRM mode with the selected precursor (protonated 1-hydroxybufuralol)/product ion pairs of m/z 278.1 → 242.0 ($[M+H\text{-}2H_2O]^+$) and 278.1 → 186.1 ($[M+H\text{-}2H_2O\text{-}C(CH_3)_3]^+$).

3.1. Enzyme kinetics

Bufuralol is a fairly specific substrate of the CYP2D6 enzyme and thus bufuralol 1-hydroxylation is often used as a model reaction to determine the activity of this CYP isoenzyme [22,23]. However, few other CYP enzymes, of which the two most important are CYP1A2 [24] and CYP2C19 [25], also exhibit minor bufuralol 1-hydroxylase activity in addition to CYP2D6. In this study, we determined the kinetic parameters (K_m and V_{max}) of bufuralol 1-hydroxylation in HLM in order to demonstrate the applicability of the SU-8 microchips to enzyme kinetics studies. The kinetic parameters of the bufuralol 1-hydroxylation in HLM were determined by the developed microchip CE-ESI/MS/MS method. As a result, the CYP mediated metabolism was shown to follow Michaelis–Menten kinetics with K_m and V_{max} values of 55 μM and 147 pmol/min/mg protein, respectively (Fig. 3). These kinetic parameters compare very well with the values determined by in-house HPLC-UV (K_m = 31 μM, V_{max} = 185 pmol/min/mg protein) and also with the literature values for K_m of 50–250 μM [24] and for V_{max} of 60–240 pmol/min/mg protein [25]. In contrast to conventional HPLC analysis, the SU-8 CE-ESI/MS microchips offer significantly improved speed of analysis and lower sample consumption. Here, the migration time of 1-hydroxybufuralol and bufuralol were only 20.6 s and 22.5 s, respectively, while the corresponding retention times with the in-house HPLC-UV method were 5.0 min and 6.5 min. In addition, the microchip CE-ESI/MS consumes only few tens of picoliters of sample (here approximately 45 pL) while a typical injected volume in HPLC is tens of μL (here 20 μL). Instead, the actual volumes of sample needed for the microchip CE-ESI/MS analysis (here 3 μL applied to the sample inlet) and the HPLC-UV analysis (here 30–40 μL in the sample vial) largely depend on the injector geometry and typically differ from each other by an order of magnitude.

3.2. Analysis of urine samples

In addition to enzyme kinetics, microchip CE-ESI/MS was used in the screening of metabolites of tramadol and paracetamol from human urine samples. The metabolic profiles of these two pharmaceuticals are very different and their broad spectrum of potential metabolites with variable chemical and physical properties make them particularly suitable reference compounds for validating the microchip CE-ESI/MS method for metabolic profiling. Namely, the main urinary metabolites of paracetamol are the phase II conjugation products, e.g., glucuronides [26] while tramadol is extensively converted to several phase I metabolites (oxidation products) by CYP enzymes. The main urinary metabolites of tramadol are *O*-desmethyl-tramadol (metabolite M1) and *N*-desmethyl-tramadol (metabolite M2) [27]. In addition, traces of several other metabolites, both phase I and phase II (conjugation products) are possible [28].

Before analysis, the urine samples were pre-treated either by off-chip SPE (5-fold concentration) or by on-chip LPME (2-fold concentration). SPE is extensively used in metabolic profiling because of its versatility and the possibility of extracting a broad range of metabolites from urine samples, for example. In this study, six tramadol metabolites, including both phase I and phase II products, as well as tramadol itself were detected and separated from each other by the microchip-based analysis following off-chip SPE (Fig. 4). In case of paracetamol, mainly phase II conjugation products (glu-

curonide, glutathione and cystein) were detected in addition to paracetamol itself which was detected as a very intense, slightly tailing peak in the electropherogram (Fig. 5). The biotransformation of xenobiotics is a very complex process and can greatly vary between individuals. Here, the content of unmetabolized paracetamol in urine was seemingly much higher than those of the metabolites. Taking into account the relatively high dose of paracetamol (500 mg), it is likely that this was the reason for the observed tailing of the paracetamol peak in Fig. 5. All tramadol and paracetamol metabolites migrated within approximately 30–35 s. These metabolite findings are in good accordance with the previously published reports [29,30].

In addition to SPE, the urine samples containing metabolites of tramadol were pre-treated using on-line LPME prior to microchip CE-ESI/MS. LPME offers selectivity in the analysis of less polar phase I metabolites, which may be hard to detect if the SPE conditions are optimized for the extraction of very polar phase II metabolites. In LPME, target analytes are extracted from the biological matrix, through a hollow fiber wetted with an organic solvent, into a suitable acceptor solution [31]. The acceptor solution is then injected into a chromatographic or electrophoretic separation system. LPME is easily downscaled to low μL volumes by replacing the hollow fiber with a flat polypropylene membrane, which also facilitates its implementation to lab-on-a-chip systems [21]. The experimental set-up used in this study is shown in Fig. 1. For extraction of the tramadol metabolites, the urine pH was adjusted to 11.4 with NaOH (30 mM) in order to convert tramadol as well as the expected basic metabolites into their neutral form (Fig. 6). In the acceptor droplet, the pH was adjusted to 2.7 with formic acid (0.1%) to ensure full protonation. In brief, the analytes in their neutral form were extracted from 4 μL of alkaline donor solution into the organic phase (octanol wetted polypropylene membrane) from which they were distributed and concentrated (2-fold) into 2 μL of acidic acceptor solution in their protonated form. The pH gradient between the donor and acceptor solution served as the only driving force for extraction, since no stirring was applied to promote mass transfer in the system. Fig. 6 lists the metabolites of tramadol detected using the LPME set-up. All compounds except the metabolite M5 could be easily detected even in full scan MS mode. For reference, the estimated detection limits (S/N = 3) of the tramadol M1 metabolite, for example, were 2 μM and 4 nM in full-scan and SRM modes, respectively. The metabolite M5 was only observed by using the more specific and sensitive detection in SRM mode, which was likely because of its poorer extraction efficiency (i.e., zwitterionic nature and relatively low log D value at pH 11, which lower its theoretical mass transfer from the donor solution into the octanol wetted membrane).

A closer examination of the SPE and LPME treated samples clearly shows the differences between the two methods. The phase I metabolites M1 and M5 as well as tramadol itself were detected by both SPE (Fig. 4) and LPME (Fig. 6). As expected, the phase II metabolites were detected by SPE only, whereas LPME provided increased selectivity with respect to the less polar phase I metabolites so that one additional metabolite, M9, was detected by LPME only. A further advantage of the LPME set-up is the possibility of performing on-line sample clean-up and preconcentration prior to analysis. In this work a preconcentration factor of two was used, but depending on the applied chip material and fabrication method, the depth of the sample inlet (i.e., acceptor side) can be reduced to only a few tens of micrometers which correspond to acceptor volumes in the nL range. Thereby, multi-fold sample preconcentration can easily be achieved with on-chip LPME in the same way as with off-chip SPE. In addition, on-chip LPME offers advantages in terms of speed of analysis. For example the time for sample preparation by LPME is only a few minutes (here a 5 min extraction time was used), while the time for sample preparation by conventional off-chip SPE columns is tens of minutes.

4. Conclusions

Sensitive and efficient analysis of drug metabolism products was demonstrated by using the SU-8 CE-ESI/MS microchips. The microchip method including rapid CE separation of parent drugs and their metabolites followed by MS detection in full-scan MS or SRM mode was validated by using 1-hydroxybufuralol, a CYP metabolism product of bufuralol, as the model compound. The enzyme kinetic parameters determined for the bufuralol 1-hydroxylation compared very well between the microchip method and a standard HPLC-UV method. The microchips were also applied to the analysis of authentic urine samples from which metabolites of tramadol or paracetamol were detected. Before microchip CE-ESI/MS analysis, the urine samples were pre-treated with either off-chip SPE or on-chip LPME. Comparison of these sample pre-treatment methods evidenced that LPME increases selectivity for the less polar phase I metabolites, while SPE is capable of extracting a broad range of phase II metabolites. However, the small sample volumes required in LPME together with the possibility of very simple on-line coupling to the separation microchips offer advantages in terms of speed of analysis and performing on-line sample concentration prior to analysis. Most importantly, the highly reproducible, low cost fabrication of SU-8 microchips by photolithography and wafer-level adhesive bonding enables mass production of microchips with accurately defined microstructures and identical features from chip to chip. In addition to the very fast analysis times and the reported high sensitivity, this is the main advantage of the fully integrated SU-8 CE-ESI/MS microchips over other thus far published CE-ESI/MS microchips.

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Short Communication

Shape-anchored porous polymer monoliths for integrated online solid-phase extraction-microchip electrophoresis-electrospray ionization mass spectrometry

We report a simple protocol for fabrication of shape-anchored porous polymer monoliths (PPMs) for on-chip SPE prior to online microchip electrophoresis (ME) separation and on-chip (ESI/MS). The chip design comprises a standard ME separation channel with simple cross injector and a fully integrated ESI emitter featuring coaxial sheath liquid channel. The monolith zone was prepared in situ at the injection cross by laser-initiated photopolymerization through the microchip cover layer. The use of high-power laser allowed not only maskless patterning of a precisely defined monolith zone, but also faster exposure time (here, 7 min) compared with flood exposure UV lamps. The size of the monolith pattern was defined by the diameter of the laser output (∅500 μm) and the porosity was geared toward high through-flow to allow electrokinetic actuation and thus avoid coupling to external pumps. Placing the monolith at the injection cross enabled firm anchoring based on its cross-shape so that no surface premodification with anchoring linkers was needed. In addition, sample loading and subsequent injection (elution) to the separation channel could be performed similar to standard ME setup. As a result, 15- to 23-fold enrichment factors were obtained already at loading (preconcentration) times as short as 25 s without sacrificing the throughput of ME analysis. The performance of the SPE-ME-ESI/MS chip was repeatable within 3.1% and 11.5% RSD ($n = 3$) in terms of migration time and peak height, respectively, and linear correlation was observed between the loading time and peak area.

Keywords:
Electrospray ionization / Mass spectrometry / Microchip electrophoresis / Porous polymer monoliths / Solid-phase extraction DOI 10.1002/elps.201400278

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Integration of multiple analytical operations on a single microfluidic chip via miniaturization is one of the forefront technologies for modern bioanalysis. The combination of microchip (capillary) electrophoresis (ME) with on-chip (ESI/MS) is a particularly powerful tool because of its high separation efficiency and selectivity of ME and MS, respectively [1–4]. As a result of miniaturization, multiple repeated analyses can be performed out of a few microliters sample volume, since only a few tens of picoliter is effectively used (injected) for a single analysis. At best, detection limits reaching low attomole amounts per injected volume (typically submicromolar concentration limits) have been shown [5–7]. However, there is a need for coupling online sample pretreatment as an integral part of the separation chips in order to improve the efficiency of sample consumption (injected vs. total sample volume required) and to reach better concentration sensitivity [8].

Although electrokinetic sample stacking [8] and liquid–liquid extraction [7, 9, 10] have efficiently been implemented on chip, SPE typically allows for improved selectivity and enrichment factors in bioanalysis. To accomplish SPE on a microchip, solid support structures have to be incorporated

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Abbreviations: ME, microchip (capillary) electrophoresis; **PPM**, porous polymer monolith

Colour Online: See the article to view online Fig. 2 in colour.

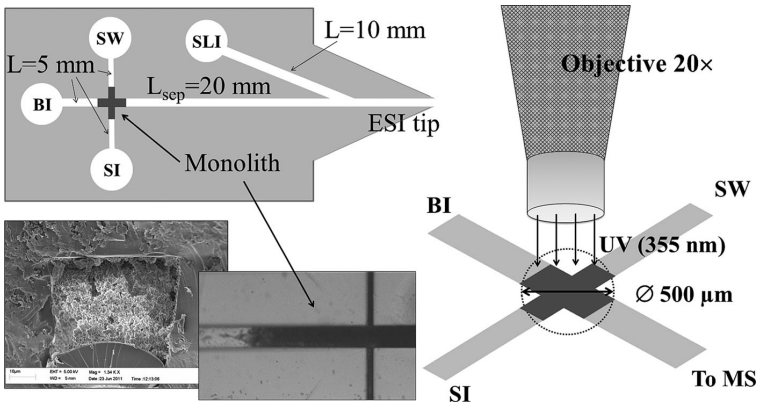

Figure 1. Schematic views of the SU-8-based ME-ESI chip with a UV-cured PPM pattern at the injection cross (top left, dimensions not to scale) and of the UV curing setup used for crosslinking of the cross-shaped monolith zone at the injection cross (right) accompanied by an SEM image and a photograph (topview) of the microchannel cross section filled with cross-linked porous methacrylate monolith (bottom left, porogen PEG10k, exposure time 15 min). BI = buffer inlet, SI = sample inlet, SW = sample waste, SLI = sheath liquid inlet, MS = mass spectrometer.

into the microchannel [11, 12], which has been achieved in several different ways, for example, coating of the channel wall with octadecyltrimethylsilane [13] or using magnetic or mechanical trapping of C-18 beads [14–19]. Precisely ordered microfabricated silicon pillar arrays have also been used as alternatives to packed beads [20, 21], but their selectivity is often limited and postmodification has to be performed in order to add stationary phase material onto the silicon pillars.

Porous polymer monoliths (PPMs) offer several attractive features for microchannel packing for on-chip SPE, including fritless polymerization in situ by heat or UV light, and easy tuning of the porosity and the surface chemistry by simply changing the polymerization time and/or monomer/porogen composition [22]. In addition, cross-linked polymer monoliths avoid the risk of individual beads leaking out of the microchannel, which is particularly important when coupling to an MS instrument is desired. However, surface prefunctionalization of the channel walls with suitable linker molecules is often required in order to permanently fix the cross-linked monolith zones into a microchannel.

In this work, we report a simple and fast approach for implementing a PPM-based SPE unit as an integral part of a standard ME separation chip made of SU-8 polymer [23] and featuring an on-chip ESI emitter with a coaxial sheath liquid channel for coupling to MS (Fig. 1). The novel approach herein arise from the use of high-power UV laser for maskless photopolymerization of a precisely defined, cross-shaped PPM zone in situ at the injection cross of the SU-8 separation chip (Fig. 1). The high intensity of the laser beam (15 µJ at 1 kHz, 355 nm) enables very fast curing (here, best performance at 7 min) and initiation of the photopolymerization reaction even through a 70-µm-thick SU-8 cover layer, which itself absorbs the near UV light to some degree [24]. To our knowledge, most previous work have made use of lower power flood exposure lamps, which entails the use of photoexposure masks and UV transparent substrates only, and also requires relatively long exposure times (typically 20–40 min [25, 26]). Therefore, our approach provides significant simplicity for fabrication of PPMs inside a microchannel. Most importantly, the placing of the cross-linked monolith at the intersection of the injection and separation channels (Fig. 1) allowed us to firmly anchor the monolith based on its shape with no need for prefunctionalization of the SU-8 surface with molecular linkers. Since the UV laser was coupled to an (upright) epifluorescence microscope, the size of the exposure beam, and thus the size of the cross-linked monolith pattern, could be adjusted by simply changing the microscopic magnitude. In this work, a 20× magnification was used in order to define a 500-µm-diameter exposure area. Before exposure, the microchannel was filled with a methacrylate monomer mixture containing appropriate crosslinkers and porogens, according to a previously published recipe [27] (see Supporting Information). Distinct from the previous work, we used a fluorescent-labeled porogen (PEG 5k) for monitoring and ensuring complete removal of PEG residues (after crosslinking), which is crucial regarding the MS coupling as PEG residues cause major background disturbance in MS analysis (see Supporting Information). Finally, the morphologies and the performance of the monoliths were characterized by SEM (Fig. 1) as well as fluorescence microscopy and ME-ESI/MS experiments (see Supporting Information).

Regardless of the microchannel packing technique, most chip-based SPE devices thus far reported have been coupled to external pumps in order to allow for operation by pressure-driven flow. Obviously, the use of pressure flow makes the system much more complex as interfacing between microchips and external pumps often requires implementation of pressure-tight capillary couplings and mechanical valves and thus makes the system prone to dead volume (time lag). In this work, with a view to performing rapid online preconcentration, the porosity of the monolith was compromised with relatively high through-flow (i.e., large pores for low back pressure) over flawless retention in order to allow for fully electrokinetic actuation and thus avoid the need for coupling to external pumps. In this respect, the PPMs appeared as a more convenient technology over particle packing because of the possibility to tune the porosity of the monolith by simply changing the amount and size of porogens and

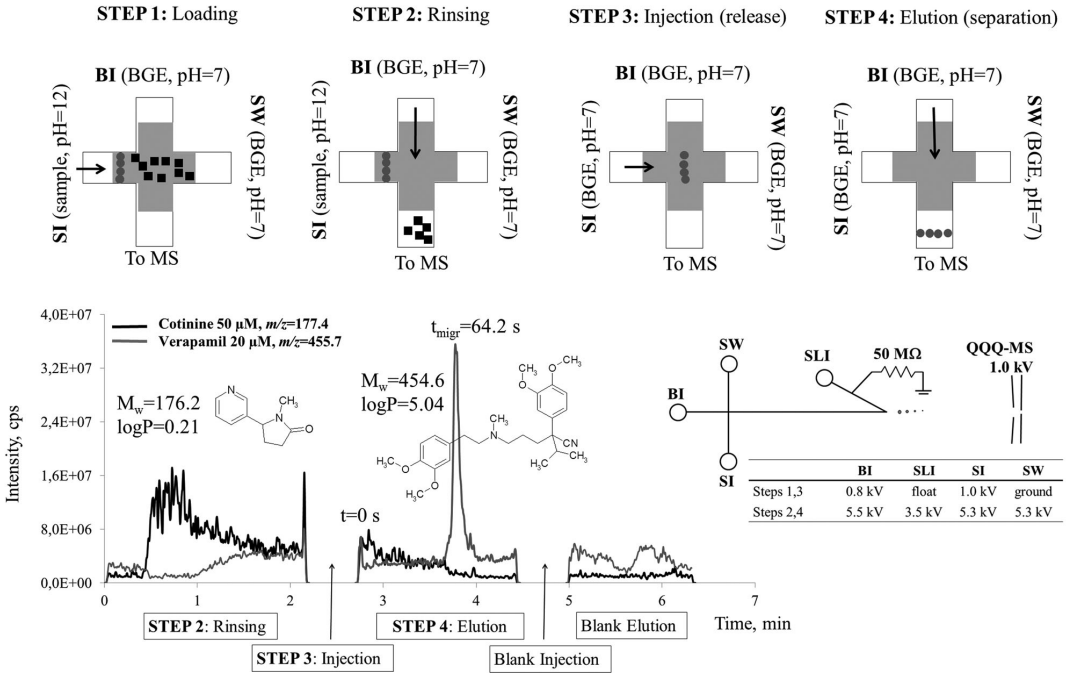

Figure 2. The operation principle of the SPE-ME-ESI microchip is illustrated with squares (black) and circles (red) representing hydrophilic and hydrophobic sample components, respectively. EIE of the $[M+H]^+$ ions of cotinine (black line, 50 μM) and verapamil (red line, 20 μM) show that the hydrophilic, unretained cotinine is washed away in step 2, whereas hydrophobic verapamil is selectively retained (steps 1–2), released (step 3), and eluted to MS (step 4). The electric field strength during separation was 800 V/cm and other operating voltages (steps 1–4) were according to the schematic view. The blank injection (~4.5–5 min) and elution (~5–6.5 min) in the end of the electropherogram show no memory effect, that is, all sample components are efficiently released from the monolith in steps 3–4. The sample components were loaded in 2% ammonium hydroxide solution (pH 12) and the BGE was 30 mM ammonium acetate with 50% methanol.

the exposure time. The operation principle of our fully integrated SPE-ME-ESI chip is presented in Fig. 2 and illustrated with a four-step protocol: (1) sample loading, (2) rinsing (of unretained, hydrophilic impurities), (3) injection (release of retained hydrophobic analytes), and (4) elution followed by simultaneous ME separation (of released hydrophobic analytes) prior to online ESI/MS detection. Detailed description of the ME voltages and MS parameters is given in the Supporting Information.

In order to visualize the rinsing step, a hydrophilic compound, cotinine ($\log P = 0.21$), was added to the sample solution containing verapamil ($\log P = 5.04$) in 2% ammonium hydroxide solution (pH 12) (step 2, Fig. 2). As a result, the hydrophilic cotinine was not adsorbed on the porous monolith during sample loading (step 1), but rather spread throughout the monolith and gave a broad and tailing peak when rinsed to the MS (step 2). Instead, the more hydrophobic verapamil was concentrated at the monolith border (step 1) and released only after elution with organic solvent (50% methanol with 30 mM ammonium acetate, steps 3 and 4). Despite the relatively long loading time of 30 s, verapamil gave a narrow and symmetric peak ($w_{1/2} \sim 3$ s) at the MS detector (step 4) evidencing efficient retention and selective elution during loading and injection-separation steps, respectively. Most importantly, there was no memory effect of either cotinine or verapamil in the subsequent blank injection (SI → SO) and separation (BI → MS) steps performed immediately after analysis (Fig. 2). Also, the good repeatabilities for both the migration time (RSD = 3.1%, $n = 3$) and the peak height (RSD = 11.5%, $n = 3$) of the preconcentrated verapamil suggest robust performance from run to run. These values are comparable to those previously reported for similar ME-ESI/MS analyses, but without SPE concentration, by our group [7, 23] and others [15, 28], which clearly indicates that addition of on-chip SPE did not significantly interfere with the ME separation. Furthermore, correlation was established between the loading time (10–25 s) and the peak area obtained using two test compounds with somewhat similar hydrophobicity, namely tramadol with $\log P = 2.45$ ($R^2 = 0.950$) and propranolol with $\log P = 2.58$ ($R^2 = 0.999$) (Fig. 3A). The estimated enrichment factors were approximately 15-fold (tramadol) and 23-fold (propranolol) already at a loading time of 25 s. The possibility to use such short loading times (which are comparable to ME injection times) emphasizes the fact that our chip design

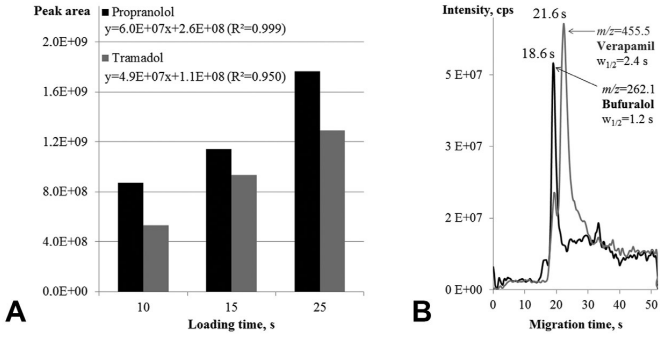

Figure 3. (A) Effect of the sample loading time (step 1) on the area of the released peaks (step 3) as illustrated with tramadol (log$P$ = 2.45) and propranolol (log$P$ = 2.58). (B) Separation of preconcentrated bufuralol (log$P$ = 2.99; black line) and verapamil (log$P$ = 5.04; grey line) after simultaneous loading into the monolith (step 1, 15 s), release (step 3), and separation by ME (step 4). Sample loading (each 25 μM) and rinsing (steps 1–3) was according to Fig. 2, but the electric field strength during separation (step 4) was 900 V/cm.

enables efficient SPE without compromising the inherent high speed of ME separations as illustrated with rapid SPE-ME-ESI/MS analysis of preconcentrated pharmaceuticals in Fig. 3B.

In conclusion, we report a simple and rapid fabrication protocol for implementation of on-chip SPE based on maskless laser-initiated photopolymerization of PPMs in situ at the injection cross of an ME-ESI chip (made of SU-8). In this work, conventional RP chemistry (of metacrylate monoliths) was exploited to SPE, but the selectivity of the monolith can easily be altered by simply changing the monomer composition. The porosity of the monolith zone was geared toward relatively high through-flow in order to reduce the back pressure, and thus allow actuation based on electrokinetic flow alone. This was considered advantageous for precise flow control and easy valving with negligible dead volume (time lag) as compared to pressure-driven flow. Anchoring of the monolith based on its shape eliminated the need for surface prefunctionalization with anchoring linkers and this made the fabrication process particularly straightforward. The use of shape-anchored PPMs was also preferred over packed beads in order to avoid the risk of individual beads leaking to the separation channel, which could cause problems in the MS measurements. Apart from this work, only a few prior examples of similar electrokinetically operated on-chip SPE with subsequent online ME have been reported [25,26,29,30]. However, in most of these devices, the loading/preconcentration time required for SPE has been rather long (5–20 min) compared to our design and coupling to MS detection has been rare. In our chip design, all analytical operations including online coupling to MS via a fully integrated, dead-volume-free ESI emitter – are combined on a single ME-ESI chip featuring relatively simple fluidic layout so that the microchips can be mass produced in a reproducible manner based on standard parallel microfabrication processes (lithography and wafer-scale adhesive bonding). The PPM, in turn, can be prepared on demand whenever sample pretreatment by SPE is considered necessary prior to ME-ESI/MS analysis. Most importantly, the chip configuration used in this study allows for online SPE with the standard ME chip geometry and with only four individual high voltage (HV) supplies so that the complexity of the system is not increased at the cost of integrated sample preparation. To our knowledge, this is the first fully electrokinetically actuated online SPE-electrophoresis microchip coupled to on-chip ESI/MS. For future work, 2D separations are envisioned through selective retention and release of sample components from the monolith (first dimension) followed by subsequent electrophoretic separation during the elution step (second dimension).

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The authors have declared no conflict of interest.

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Supporting Information

Shape-Anchored Porous Polymer Monoliths for Integrated Online Solid-Phase Extraction-Microchip Electrophoresis-Electrospray Ionization Mass Spectrometry

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Keywords: electrospray ionization, mass spectrometry, microchip electrophoresis, porous polymer monoliths, solid phase extraction

Materials and Reagents

Verapamil hydrochloride, propranolol hydrochloride, cotinine, ammonium acetate, 2,3-epoxypropyl methacrylate (GMA), 2-methoxyethanol (2ME), benzoin methyl ether (BME), 1-methyl 2-pyrrolidone (NMP), trimethylolpropane trimethacrylate (TRIM), and triethylene glycol dimethacrylate (TEGDMA) were purchased from Sigma-Aldrich (Steinheim, Germany). Polyethyleneglycol 6000 (PEG (6k)) was from Applichem (Darmstadt, Germany) and fluorescein polyethyleneglycol succinimidyl ester 5000 (fluorescein-PEG-NHS- 5k) was from Creative PEGWorks (Winston Salem, NC). Methanol, acetic acid, and ammonium hydroxide were from Mallinckrodt Baker B.V. (Deventer, The Netherlands). Tramadol was kindly donated by the Department of Forensic Medicine, University of Helsinki, Finland. Water was purified with a Milli-Q water purification system (Millipore, Molsheim, France). All solutions were sonicated for 15 min before use.

Negative photoresist SU-8 50 (Microchem, Newton, MA, USA) was from Microresist Technologies GmbH (Berlin, Germany) and poly(dimethylsiloxane) (PDMS) (Sylgard 184, Dow Corning, Midland, MI, USA) from VWR International Oy (Espoo, Finland).

Microchip design

The microchips used in this study comprised a 25-mm-long (20 mm effective length) separation channel (50×50 µm, w×h) intersected by a 10-mm-long simple cross injection channel (30×50 µm, w×h), an auxiliary channel (100×50 µm, w×h) for sheath liquid, and an ESI emitter (Fig. 1). The microchips were fabricated of epoxy photoresist SU-8 using photolithography and adhesive bonding techniques as reported earlier [1, 2]. Prior to use, thin sheets of PDMS with Ø2 mm inlet holes were attached on top of the microchip to avoid liquid spreading on SU-8 surface.

Monolith Preparation

The methacrylate monolith was prepared according to a protocol slightly modified from that previously published [3, 4]. Briefly, the monomer mixture was prepared by mixing the main monomer GMA with the crosslinkers TRIM and TEGDMA in a mass ratio of 1:1 (TRIM:TEGDMA ratio 1:5, w/w). Next, the main porogen PEG dissolved in 2ME (1:5, w/w) and the co-porogen NMP was added to the monomer mixture. The main porogen used during the method development was fluorescein-labeled PEG (5k) which allowed us to monitor the efficiency of washing based on the fainting fluorescence. However, for the final monolith preparation, non-fluoresent PEG (6k) was used. The final ratio of the components in the mixture was 30% monomers and cross-linkers, 60% main porogen, and 10% co-porogen. Last, the photoinitiator BME was added to the polymer solution to a final concentration of 1% (w/w). The mixture was vortexed and sonicated for 10 minutes after which the microchip was filled with the solution. Polymerization was done by exposing the injection cross area through the SU-8 cover layer (thickness 70 µm) with a UV laser (355 nm, 15 µJ at 1 kHz, PowerChip, Teem Photonics) for 7 minutes (Figure 1). The UV laser was coupled to a Zeiss Axioscope A1 upright epifluorescence microscope (Carl Zeiss Oy, Espoo, Finland) through a Plan-Neofluar 20×/0.30 objective so that the exposed area was roughly 500 µm in diameter. After exposure, the microchip was filled with and immersed in a water:methanol (1:1) solution at 60°C for 1 h to dissolve the unpolymerized methacrylate monomers. In addition, the microchannel was rinsed by vacuum suction for 10 minutes before and after immersion in the warm water:methanol solution in order to efficiently remove the dissolved porogens from the microchannel. The morphology of the monolith was characterized by scanning electron microscope (SEM) photographs (Figure 1, see manuscript).

Microchip electrophoresis and Mass Spectrometry

Schematic illustration of the operation principle of the integrated on-chip SPE-MCE-ESI/MS is presented in Figure 2 (see manuscript). The SPE-ME-ESI/MS chip was placed on an *xyz*-aligning stage in front of an API3000 or API365 triple-quadrupole MS (Perkin-Elmer Sciex, Concord, ON, Canada). An external power supply (Micralyne Inc., Edmonton, Canada) was used for application of the loading/injection, rinsing/separation and ESI voltages through platinum wires placed in the microchip inlets. The MS was operated in positive ion mode with an ESI voltage of 3.5 kV applied to the sheath liquid inlet (SLI). The ESI voltage also served as the counter voltage for the ME separation. Before analysis, the monolith was equilibrated with the background electrolyte (BGE) which consisted of 30 mM ammonium acetate with 50% methanol (v/v). Sample loading (in 2% ammoniumhydroxide, pH 12) and injection (in BGE) were performed in pinched mode with the following voltages: 1.0 kV (sample inlet (SI)), ground (sample waste (SW)), and 0.8 kV (buffer inlet (BI)) (Figure 1). The ME separations were performed in cathodic mode using a separation voltage of 5.5 kV applied to the BI (800 V/cm between the BI and the SLI) and antileakage voltages of 5.3 kV applied to the SI and SW, while an ESI voltage of 3.5 kV was applied to the SLI. The sheath liquid used for all analyses was methanol:water 80:20 (v/v) with 1% acetic acid. The excess separation current was grounded through a 50 MΩ resistor coupled in parallel with the ESI voltage supply in a configuration similar to earlier work [5]. Data were recorded in full-scan MS mode at a speed of 300 ms per scan over a mass range of *m/z* 50-500. Analyst 1.4 software was used for data acquisition and processing.

Characterization of the Monoliths

Rinsing of the porogens and unpolymerized methacrylate monomers from the monolith was found to be crucial in order to minimize background disturbances and suppression of ionization in ESI/MS. Therefore, the washing procedure was optimized by using fluorescent labeled porogens (fluorescein-PEG5k) so that the washing efficiency could be monitored based on the fainting fluorescence of the monolith pattern, in addition to monitoring the quality of the mass spectra (Fig. S1). Here, a one-hour water-methanol 1:1 bath at +60°C with additional rinsing by vacuum suction for 10 minutes before and after the bath was found adequate in order to assure high quality MS analysis (Fig. S1). Namely, abundant PEG residues were observed unless the washing procedure was carefully optimized with help of fluorescent-labeled PEG.

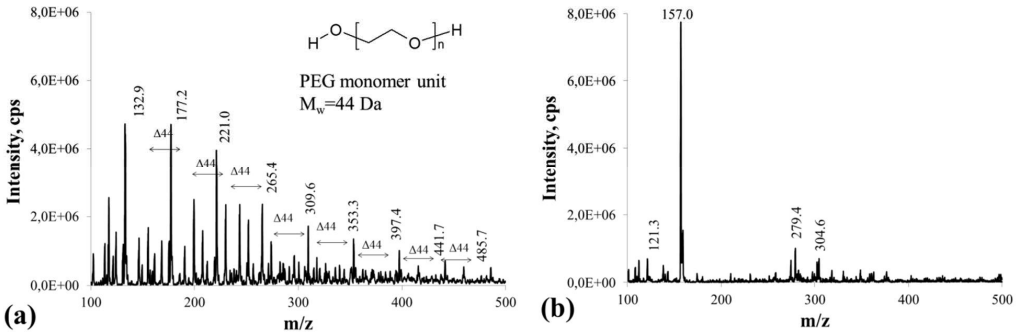

Figure S1. ESI/MS spectra of a BGE run obtained by using an SU-8 ME-ESI/MS chip with porous methacrylate pattern at the injection cross: (A) after inadequate washing showing the characteristic PEG patterns and (B) after a one-hour water-methanol 1:1 bath (60°C) and additional rinsing by vacuum suction for 10 min showing negligible number of different background ions.

In addition to monolith washing, fluorescence microscopy was exploited to examining the retention capacity of the monolith. A fluorescent dye of similar lipophilicity than the test compounds (i.e., rhodamine, logP=2.85) was used for illustration of the analyte adsorption onto the monolith (Fig. S2). It was observed that the fluorescent dye was firmly retained to the metacrylate monolith during sample loading (Step 1) and did not elute when rinsing the monolith with aqueous buffer (Step 2), but until organic solvent was added to the run buffer for sample injection/release (Steps 3-4, data not shown).

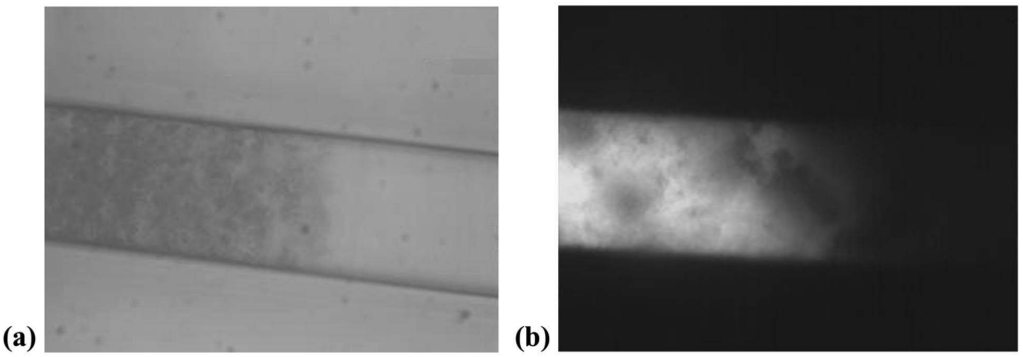

Figure S2. (a) A micrograph (topview) of a microchannel filled with monolith and (b) adsorption of a fluorescent dye (10 µM rhodamine 123) onto the monolith during sample loading in 100 mM Tris (pH 9).

Although the metacrylate monoliths as such showed reversed phase chemistry, it was obvious that some sample leakage may occur between the monolith pattern and the microchannel wall since the monolith patterns were not cross-linked to the microchannel surface but rather anchored based on their shape. In order to examine the effect of sample leakage (during loading) on the quantitativity of sample enrichment, a series of SPE-ME-ESI/MS runs was performed with varying sample loading times. It was observed that, if there were void volumes in the

monolith (e.g., at microchannel corners or near microchannel walls), minor sample leakage took place during loading so that traces of the test compounds were eluted to the MS already during the rinsing step. However, the amounts (peak areas) of leaking sample components hardly exceeded 10% of those obtained after controlled release (injection and elution) of the same sample components from the monolith (Fig. S3). Nevertheless, the SPE chip showed quantitative performance and linear regression between the loading time and the enrichment factor was obtained despite of the minor sample leakage. Since the emphasis here was put on developing prominently rapid sample preparation protocols, and not high performance separations, the minor leakage during sample loading did not play a crucial role with respect to the chip performance. Instead, the possibility to use electrokinetic flow instead of pressure driven flow was highly beneficial in terms of minimizing the time required for SPE as no capillary couplings to external pumps (with inherent dead volume/time lag) were needed. Thus, relatively loose monoliths with high degree of porosity are likely the best compromise in order to reduce the back pressure (macro pores), but still maintain sufficient retention capacity (micro pores).

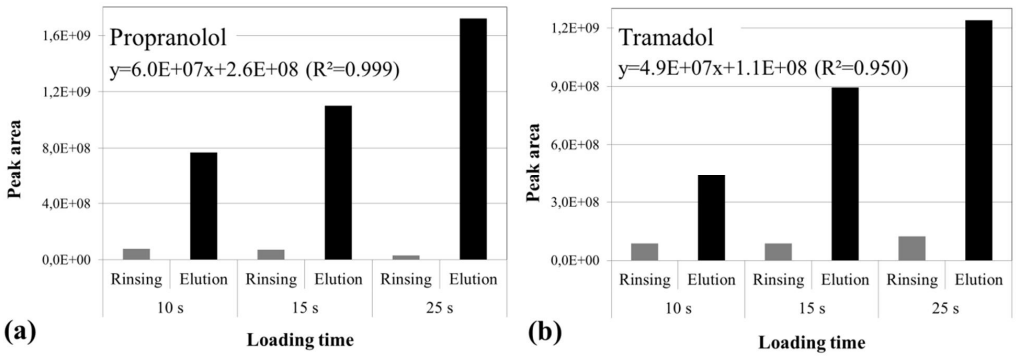

Figure S3. Comparison of the peak areas observed for (a) propranolol and (b) tramadol signals originating from sample leaking during loading (i.e., Step 2, rinsing) and from controlled sample release (i.e., Step 4, elution).

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